The Plasma Proteome of Children Recovering From Severe Malarial Anaemia

Thesis

THE PLASMA PROTEOME OF CHILDREN RECOVERING FROM SEVERE MALARIAL ANAEMIA

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DECLARATION

I, Samuel Eneọjọ Abah, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis. All collaboration, supervision and assistance have been acknowledged.

Samuel Eneọjọ Abah

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DEDICATION

This Work is dedicated to all the children in the study cohort and their parents/guardians.

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LIST OF ABBREVIATIONS

ACN, Acetonitrile

AHSP, Alpha Haemoglobin Stabilizing Protein

APS, Ammonium Persulphate

ATM, Amplicon Tagment Mix

BAM, Binary Alignment/Map

BBB, Blood Brain Barrier

BCA, Bicinchoninic Acid

BME, β-mercaptoethanol

BSA, Bovine Serum Albumin

BWA, Burrows-Wheeler Aligner

CC, Community Control

CD, Cluster of Differentiation

CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate

CK, Creatine Kinase

CLU, Clusterin

CM, Cerebral Malaria

CRP, C-reactive protein

CRSR, cysteine-rich scavenger receptors

CyDye, Cyanine Dye

DAL, Diluted Amplicon Library

DIGE, Two-Dimensional-Difference-Gel Electrophoresis

DMF, Dimethylformamide

DMSO, Dimethyl Sulfoxide

DTT, Dithiothreitol

EDRF, Endothelium-derived relaxing factor

ELISA, Enzyme-linked Immunosorbent Assay

EPO, Erythropoietin

fHb, Free Haemoglobin

GRCh37, Genome Refrence Consortium Human Genome build 37

Hb, Haemoglobin

hg19, Human Genome

HNE, 4-Hydroxynonenal

HO-1, Haemoxygenase-1

Hp, Haptoglobin

HPR, Haptoglobin Related Protein

Hpx, Hemopexin

HT1, Hybridization Buffer

IEF, Isoelectric Focusing

IgG, Immunoglobulins

IGV, Integrative Genome Viewer

ILs, Interleukins

Indel, Insertion Deletion

IPG, Immobilized pH Gradient

IVH, Intravascular Hemolysis

kDa, Kilodalton

LC-MS, Liquid Chromatography–Mass Spectrometry

LDH, Lactate Dehydrogenase

LDL, Low Density Lipoprotein

LNA1, Library Normalization Additive1

LNB1, Library Normalization Bead-1

LNS1, Library Normalization Storage buffer 1

LNW1, Library Normalization Wash1

LPS, Lipopolysaccharide

MMP, Matrix Metalloproteinase

MRDT, Malaria Rapid Diagnostic Test

NPM, Nextera PCR Master mix

NT, Neutralize Tagment buffer

OPD, *o*-Phenylenediamine

PAL, Pooled Amplicon Library

PBS, Phosphate-Buffered Saline

PCR, Polymerase Chain Reaction

PCV, Packed Cell Volume

PD, Parasite Density

pfHRP2, *Plasmodium falciparum* Histidine Rich Protein-2

RBC, Red Blood Cells

RNS, Reactive Nitrogen Species

ROS, Reactive Oxygen Species

RSB, Resuspension buffer

SAM, Sequence Alignment/Map

sCD, Soluble Cluster of Differentiation

SDS-PAGE, Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

SDS, Sodium Dodecyl Sulfate

sELISA, Sandwich Enzyme-linked Immunosorbent Assay

SEVM, Severe Malaria

SMA, Severe Malarial Anaemia

SNP, Single Nucleotide Polymorphism

TACE/ADAM17, TNF-α Converting Enzymes/Disintegrin Metalloproteinase-17

TAE, Tris base, Acetic acid and Ethylenediaminetetraacetic acid (EDTA)

TD, Tagment DNA buffer

TEMED, Tetramethylethylenediamine

TMB, 3,3', 5,5'-Tetramethylbenzidine

TNF-α, Tumour Necrosis Factor- Alpha

Tris, Tris(hydroxymethyl)aminomethane

TWEAK, Tumour necrosis factor-like weak

UM, Uncomplicated Malaria

VCF, Variant Call Format

WGA, Wheat Gel Agglutinin

2DE, Two Dimensional Gel Electrophoresis

ABSTRACT

The majority of malaria-associated deaths occur in children less than 5 years of age and severe malaria accounts for up to 90% of such deaths. We are interested in dissecting why some children develop either one of the severe malarial syndromes or the uncomplicated Malaria (UM). We studied a total of 1,622 plasma and 519 DNA samples from our pediatric Nigerian cohort. By monitoring and comparing the differences in the plasma proteome in the UM group to each of the severe syndromes at both onset and through convalescence we found that congenitally low plasma Haptoglobin (Hp), low/stable plasma level of sCD163, sub-optimal plasma Hemopexin (HPx) level and low plasma clusterin (CLU) levels could be put forward as predictors of acute/insidious onset of severe malarial anaemia (SMA) and Cerebral Malaria (CM). We show that low plasma CLU is a very specific marker of CM at acute onset and propose a mechanism through which high CLU levels can protect from CM. We also propose that circulatory HPx level is useful as both diagnostic and prognostic biomarkers of severe malaria as its level varies among the severe malaria forms at acute onset and also increases sensitively with response to treatment in all the malaria groups.

We show that Hp phenotypes could be linked to the pathological differences that underlie the acute onset of UM, severe malaria and disease progression among subjects: Hp phenotypes seem to modulate response to malarial infection and might be responsible for contradictory reports on the pathophysiology of severe malaria. We also show for the first time that *Hp* gene sequence variants/deletions at sites in both the distal and proximal upstream region are significantly associated with protection from and susceptibility to malaria, whether or not they are associated with plasma Hp levels.

CHAPTER ONE

1.0 INTRODUCTION

1.1 The History of malaria

The scourge of malaria has co-existed with the human race since antiquity. A suspected malaria illness was reported in a Chinese document as early as 2700BC and in an ancient Egyptian Papyri from about 1570BC (Cox, 2010). During the time of Hippocrates (460-370BC), malaria was known as paroxysms of fever, it was known to progress from chills to fever to sweats and then further exacerbation. Hippocrates trying to describe malaria symptoms said, "*When paroxysms fall on even days, the crisis will be on even days; and when paroxysms fall on odd days, the crisis will be on odd days.... There may be relapse and it could be deadly*" (Cunha and Cunha, 2008). From the ancient time, the incidence of malaria has been linked to poverty and poor environment such as marshy areas (Cox, 2010). However, the causative agent was unknown. Insight into the study of malaria began when Charles Louis Alphonse Laveran discovered the malaria parasite while working in Algeria in the year 1880 (Cox, 2010). Furthermore, Ronald Ross in 1897 discovered the mosquito as a vector for avian malaria and about the same time, other scientists also reported the mosquito as a vector for human malaria (Cox, 2010).

Malaria was thought to originally affect all continents except the Antarctic (Cox, 2010, Carter and Mendis, 2002). In 1955, the WHO launched a global eradication of malaria programme through the use of DDT and other insecticides that brought about total eradication of malaria in Europe and other developed countries in 1967 (Majori, 2012, Trigg and Kondrachine, 1998). This also brought about a significant eradication of malaria in Asia and Latin America. Unfortunately, Africa did not benefit from the campaign before it ended in 1969 (Majori, 2012, acentalNajera, 2001, Trigg and Kondrachine, 1998). Other subsequent control

measures adopted by the WHO have not been very effective. Hence, the scourge of malaria in sub-Saharan Africa remains very critical (Majori, 2012, Trigg and Kondrachine, 1998, Teklehaimanot and Mejia, 2008).

This scourge of malaria in sub-Saharan Africa remains unresolved due to absence of active surveillance; lack of improvement in the health services; lack of development in both urban and rural areas (Majori, 2012); antimalarial drug resistance (Talisuna et al., 2004, Ng et al., 2015); difficulty in access to research funding in recent years (Talisuna et al., 2015), funding often appearing out of tune with political statement (Najera, 2001); absence of an effective vaccine (Tran et al., 2015, Owens, 2015); inadequate parasite and vector-based studies (Talisuna et al., 2015, Stone et al., 2015); poverty (Ricci, 2012, Teklehaimanot and Mejia, 2008, Kaler, 2008, Sylvester and Ivan, 2006, Worrall et al., 2005, Kmietowicz, 2000, 1992); lack of rapid and specific diagnosis (Singh et al., 2014, Okiro et al., 2009, Drakeley and Reyburn, 2009); lack of proper legislation on drug production, distribution and usage (Lalani et al., 2015, Chaccour et al., 2015) and emerging resistance of the vector to insecticides (Mnzava et al., 2015, Lindblade et al., 2015, Weetman and Donnelly, 2015).

Outside of Africa, wherever malaria is or has been endemic, the female mosquito vectors of malaria are reported to be zoophilic rather than anthropophilic (Carter and Mendis, 2002). This predilection of female anopheles mosquitoes to feed on humans might partly account for their selective advantage in the region (Carter and Mendis, 2002).

1.2 Malaria infection

Malaria illness is caused by *Plasmodium* species that infect the female Anopheles mosquito. The parasite is transmitted to the vertebrate host through this mosquito bite. Infection has also been reported through other means such as

blood transfusion, organ transplant, sharing of blood contaminated sharp objects and even congenitally (McGuire et al., 1994). However, the most prevalent method of transmission is through the bite of an infected Anopheles mosquito.

The five species of *Plasmodium* (P) implicated in humans are, *P. falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi* (Kantele and Jokiranta, 2010). The genus *Plasmodium* includes over 120 species with particular host restrictions and predilections to certain geographical locations. Severe cases in humans are largely caused by *P. vivax* and *P. falciparum* and are responsible for most of the infection in sub-Saharan Africa (Manning et al., 2011). *Knowlesi* malaria was originally recognized as the disease of the macaque monkey but has been reported to affect humans in Malaysia (Singh et al., 2004, Moon et al., 2013).

The most virulent of malaria parasites is *P. falciparum*. It causes most malaria-related death in humans. The infection is unique in that the parasites infected cell binds to the endothelium during the blood stage and can be sequestered in organs including the brain, liver and the placenta (Rogerson et al., 2007). It is common in Africa, particularly in Nigeria (Olivar et al., 1991).

Unlike *falciparum*, *P. vivax* is not prevalent in Africa as the majority of Africans lack the duffy antigen on the surface of erythrocytes, a receptor for *P. vivax* (Carter and Mendis, 2002), but it is common in other tropical areas outside of Africa (Moon et al., 2013). Both *P. vivax* and *ovale* infection can be difficult to eradicate because of their ability to form hypnozoites that remain dormant in hepatocytes in the liver. On the other hand, *P. malariae* does not form hypnozoites but exhibits an asymptomatic blood stage that can persist for decades (Greenwood et al., 2008a).

The malaria parasite undergoes a multistage transformation in a complex life cycle (Fig. 1.1). The sporozoites are released into the host through the bite of a female Anopheles mosquito. These sporozoites head to hepatocytes about 45

minutes after the bite and undergo asexual reproduction forming a schizont, containing numerous merozoites (Fig.1.1). The hepatic or exoerythrocytic phase is largely without physical clinical manifestations or symptoms.

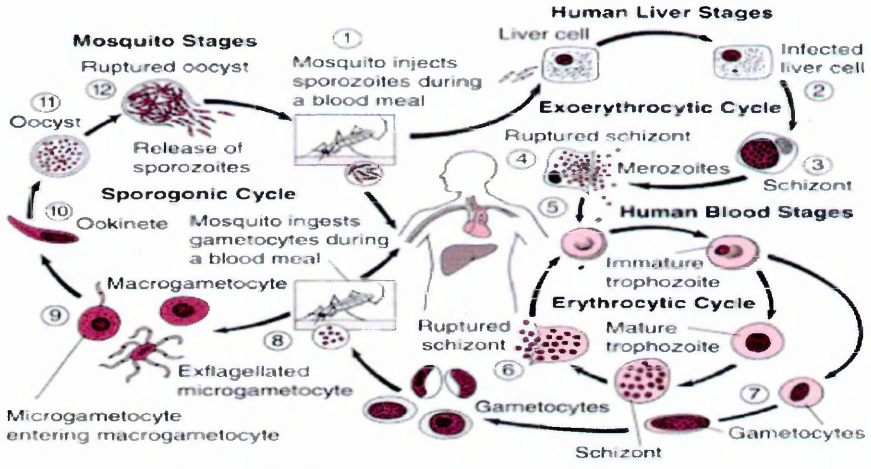

Figure 1.1 Malaria parasite life cycle

The parasite life cycle is divided into 3 major phases, the exoerythrocytic cycle, the erythrocytic cycle and the sporogonic cycle as depicted. The entire life cycle is divided into 12 stages; 1. The mosquito injects sporozoites during blood meal; 2. The sporozoites migrate to the liver; 3. Sporozoites form exoerythrocytic schizont; 4.The schizont ruptures releasing numerous merozoites; 5. The merozoites invade Red Blood Cells (RBCs) and develop through both immature and mature trophozoites to form erythrocytic schizonts. 6. The schizont ruptures releasing merozoites for continuous RBC destruction in the asexual erythrocytic phase; 7. Some merozoites form sexual gametocytes, which are picked up during mosquito blood meal and develop within the mosquito into 8. Exflagellated microgametocytes; 9. Macrogametocytes; 10. Ookinete; 11. Oocyst; and 12. Ruptured Oocyst releasing sporozoites, which are injected by the mosquito during blood meal. Source: adapted from blog; http://zitandraw.blogspot.co.uk/2011/12/life-cycle-of-plasmodium-vivax.html

The duration of the exoerythrocytic phase depends on the *Plasmodium* species; 6 days for *P. falciparum*, 8 days for *P. vivax*, 9 days for *P. ovale*, 8-9 days for *P. knowlesi* and 13-15 days for *P. malariae* (Vaughan et al., 2008). During the erythrocytic phase, the merozoites invade erythrocytes and develop through trophozoite and schizont phases in erythrocytes (Fig. 1.1). A small proportion of these asexual parasites do not undergo schizogony but differentiate into sexual stages, the male and female gametocytes (Fig. 1.1). Gametocytes are non-pathogenic but are important in the transfer of infection to another host through the bite of the vector (Fig. 1.1). The development to the gametocyte stage in *P. vivax* starts just after merozoites are released from hepatocytes and in *P. falciparum* it is

at about 7 days at the peak of asexual erythrocytic schizogony (Pukrittayakamee et al., 2008, Miller et al., 2002).

The clinical symptoms observed in malaria start when the first generation of merozoites invades erythrocytes during the erythrocytic cycle. The rupture of the schizont to release merozoites also releases parasitic products that contribute to the cause of clinical symptoms. It is also at this stage that the complex network of inflammatory cytokines begins their action (McGuire et al., 1994, Autino et al., 2012). The erythrocytic cycles occur every 24 hours in *P. knowlesi*, 48 hours in *P. falciparum*, *P. vivax* and *P. ovale* and 72 hour in *P. malariae*.

Successful parasitic invasion and survival requires a complex network of specialized proteins (Florens et al., 2002, Holder et al., 1994, Blackman et al., 1990). Each stage is associated with changes in protein expression and metabolic pathways that enable *Plasmodium* to escape immune clearance (Florens et al., 2002).

Malaria is usually associated with fever but also mimics a range of other illnesses. In areas where malaria is holoendemic, differential diagnosis of malaria is necessary to rule it out or confirm its presence due to overlapping symptoms with other diseases. Malaria usually mimics flu-like symptoms, gastroenteritis, typhoid fever, Pneumonia, meningitis, encephalitis and viral infection like hepatitis especially in children (McGuire et al., 1994, Schumacher and Spinelli, 2012). These similarities in symptoms are common to all malaria parasites but are mostly observed in *P. falciparum* malaria (Bartoloni and Zammarchi, 2012).

In Africa, there are challenges in differentiating malaria from endemic typhoid fever, particularly in areas without laboratory and trained microscopists. Osler, attempting to differentiate malaria from typhoid fever observed that despite the similarity between them such as chills, fever, headache and splenomegaly, malaria begins with multiple chills while typhoid fever begins with a single morning

chill and that chills do not characterize typhoid fever except for this initial one. Chills in malaria are accompanied by spiking fever followed by sweating, profound malaise and then complete recovery between attacks (Cunha and Cunha, 2008). However, these clinical observations have led to misdiagnosis and attempts to improve on it have been unsuccessful (Smith et al., 1994, Redd et al., 1992)

It was also observed that malaria fever is periodic depending on the length of the asexual cycle and synchronizes with the release of the erythrocytic schizont (McGuire et al., 1994, Cunha and Cunha, 2008). The time to the onset of symptoms or the detection of parasites in the blood depends on age, use of antimalarial prophylaxis, route of infection such as blood-induced infection during blood transfusion and host immunity. Strong immunity can suppress the onset of clinical symptoms; migrants from malaria holoendemic regions have been reported to have *P. falciparum* infection and remained asymptomatic for 2-8 years (Krajden et al., 1991, Greenwood et al., 2008b, Szmitko et al., 2009).

1.3 Epidemiology of malaria in sub-Saharan Africa, Nigeria.

Malaria is a global threat, particularly in Africa (Murray et al., 2014). Malaria is a major cause of ill health globally. Nearly half of the world population spanning 108 countries is exposed to malaria. The global annual malaria associated death was reported to be more than a million from 2006 to 2012 (Birhanie, 2016, Chen et al., 2016, Schumacher and Spinelli, 2012, Srivastava et al., 2015), however, some have reported about 0.6-0.5 million deaths per year between 2013 to 2015 (Kang et al., 2016, Tan et al., 2016).

While progress seems to have been made on the global platform within the last decade, much still needs to be done in sub-Saharan Africa. About 80-90% of global malaria associated deaths occurs in the Sub-Saharan Africa (Tan et al., 2016), hence not much progress seems to have been made in this region. More

than 0.6 million children that are less than 5 years of age are reported to die from malaria in sub-Saharan Africa (Schumacher and Spinelli, 2012, Huang et al., 2015) and this is mostly due to Severe Malaria Anemia (SMA) and Cerebral Malaria (CM), which account for about 90% of these deaths (Gunst et al., 1998, Delanghe et al., 1998b). SMA is the major cause of death particularly in children that are less than five years of age, pregnant women and the immune-compromised (Burte et al., 2013).

Malaria transmission is endemic and stable in Nigeria. A total of 110 million cases of malaria are clinically diagnosed per year in Nigeria (Han and Peng, 2015). Others reported that malaria accounts for up to 60% of outpatient visit in Nigeria, with about 25-30% mortality in under 5 years of age (Singh et al., 2014). Children under the age of 5 in Nigeria can have up to four malaria attacks in a year (Singh et al., 2014). Malaria is the most common cause of hospital attendance in Nigeria with an estimated annual economic loss of over US$ 835 million from the cost of treatment and other malaria associated losses (Han and Peng, 2015). About 96-98% of malaria infections in Nigeria are caused by *Plasmodium falciparum* while infections due to *Plasmodium malariae* are rare and are as low as 2-4% (Olivar et al., 1991).

While CM is the most common severe form of *P. falciparum* malaria in the northern part of Nigeria, SMA is more common in the south (Anumudu et al., 2007). A six-year (2000-2005) review show that out of 16,031 children admitted at the University College Hospital (UCH), Ibadan showed that severe malaria cases constituted 11.3% with 89.1% being children less than 5 years old and CM accounted for 19.7% of all severe malaria cases. The yearly morbidity rate from severe malaria in Ibadan, Nigeria ranged from 8.7% to 13.2% with a significant continuous increase from 2000 to 2004 (Orimadegun et al., 2007).

The epidemiological factors that enhance the development of SMA in

Nigerian children include lack of prompt response at the onset of the disease; misinterpretation of symptoms at onset; excessive use of home remedies; inappropriate use of drugs; fake drugs and non-adherence to antimalarial drug prescription refusal to attend hospital owing to tedious protocol and long waiting time; attendance at the hospital is only when symptoms seem to aggravate. This was particularly observed in the southern part of Nigeria where herbal home remedies for malaria are practiced widely and attendance at hospital is only when such home remedies have failed; (Anumudu et al., 2007).

CM is more prevalent in children between the age of 3 to 6 years while SMA is more prevalent in younger children of 0.6 to 2 years of age (Biemba et al., 2000). Age is an important predisposing factor in the development of SMA (Imbert et al., 1997). However, the intensity of transmission was thought to influence the age at which severe malaria develops; the median age of 1, 3 and 5 years was observed in regions with high, moderate and low transmission, respectively.

Reports concerning the association of age and severe malaria manifestation are contradictory. While one study reported that age and intensity of transmission are both independent variables in severe malaria (Reyburn et al., 2005), another group reported that increasing intensity of transmission caused a shift in the burden of CM but not SMA and respiratory distress towards a younger age (Roca-Feltrer et al., 2010). Plasma *falciparum*-specific antibody to malaria did not show any disparity in the different age groups (Erunkulu et al., 1992). The onset of CM is drastic and dramatic unlike the more mild and subtle onset of SMA. As a result, the appropriate management of SMA is often delayed and initially overlooked, and is a contributing factor to its prevalence.

1.4 Clinical manifestation of malaria

Just the fever cycle in the presence of parasitaemia is commonly categorized as mild or uncomplicated malaria. However severe complications of malaria constitute anaemia, and in severe anaemia the indicated haemoglobin level is less than 5g/dl; convulsion; respiratory distress; hypoglycemia; haemoglobinuria; lactic acidosis; renal failure; hypotension; shock and jaundice. Most of these complications are manifest in Severe Malarial Anaemia (SMA), Severe Malaria (SEVM) and Cerebral Malaria (CM) (Trampuz et al., 2003, Njuguna and Newton, 2004, Okeyo et al., 2013).

1.41 Anaemia in malaria

Anaemia is one of the complications of malaria due to cycles of Red Blood Cell (RBC) destruction by the parasites. Lower levels of haemoglobin concentration in people living in tropical countries compared to those in temperate regions are mostly attributed to malaria. The intensity of anaemia is usually associated with parasite density (Everaert et al., 1998c). A haemoglobin concentration lower than the mean by two standard deviations (2 SD) when adjusted for age, sex and the state of an individual including pregnancy could be considered as anaemia (McGregor et al., 1966). Generally, a haemoglobin concentration less than 11-8g/dl is regarded as anaemia. Hb levels that are less than 8g/dl or a haematocrit of less than 24% have been reported in the presence of parasitaemia (Crawley, 2004). In severe cases of malaria, the haemoglobin concentration could be less that 5g/l or a haematocrit of 15% (Okeyo et al., 2013). The presence of parasitaemia is used to differentiate malarial anaemia from anaemia of other aetiology such as poor nutrition, haemoglobinopathies (Abdalla and Pasvol, 2004), and infectious diseases (Crawley, 2004).

Anaemia in malaria, particularly P. falciparum malaria can occur even without obvious clinical symptoms in the patients (Kurtzhals et al., 1999). Haemoglobin concentration could be lower in a P. falciparum-infected asymptomatic individual by 1.0-2.0g/dl compared to healthy parasite-negative individuals. Pregnant women with asymptomatic malaria have recently been reported to have anaemia (Nega et al., 2015).

1.42 Parasite density and anaemia

The fall in haematocrit sometimes does not parallel the parasite biomass particularly during convalescence. The degree of anaemia is sometimes not equal to the severity of the infection. In acute intense infection, the Hb level might be normal despite high parasite biomass (White et al., 1992, Mackintosh et al., 2004). However, the discrepancy between Hb level and parasitaemia was suspected to be due to the sequestration of parasites in the microvasculature of vital organs. As a result, it was thought that the lower parasite levels in peripheral blood are not representative of the total body parasite biomass (White et al., 1992). As a result, it was suggested that the parasite biomass should only be computed by multiplying parasitaemia by blood volume only when sequestration is not suspected, as with benign malaria (White, 1997).

A more recent study has indicated that the accurate way of quantifying the total parasite biomass is to measure the *P. falciparum* Histidine Rich Protein 2 (PfHRP2) (Karl et al., 2015); Higher sequestration index, total parasite burden but not circulating biomass have been reported to be associated high plasma PfHRP2 (Huang et al., 2015, Karl et al., 2015).

The periodicity of both schizogony and merogony, which are characterized by the presence of numerous newly invaded ring forms could affect the number of circulating parasites in the peripheral blood (Sherman, 1998, White and Ho, 1992).

Sequestration may not be suspected when mature parasites are predominant in the peripheral blood. Therefore, assessing the stage of parasite maturity on the slide during admission could also be an indicator of malaria severity (Silamut and White, 1993, Sherman, 1998). The removal of non-infected erythrocytes carrying parasite antigen (Balla et al., 1991, Molitor et al., 1991) could also explain the disparity between Hb level and parasite density. However in some individuals, the haematocrit can be normal at the peak of infection, as earlier mentioned (White et al., 1992, Mackintosh et al., 2004). Therefore, the mechanisms behind anaemia are poorly understood and raise many questions on the pathophysiology of SMA in particular.

1.43 Severe malaria anaemia (SMA)

SMA is defined as Hb concentration that is less than 5-6g/dl in the presence of the asexual form of the parasite, without any specified parasite density (Mackintosh et al., 2004, Cox et al., 2013, Okeyo et al., 2013). *P. falciparum* malaria is the major cause of SMA, but other studies have also implicated *P. knowlesi* and *P. vivax* (McGuire et al., 1994). However, *P. falciparum* has a higher mortality rate because the parasite has a predilection for erythrocytes of all ages unlike *P. vivax* and *P. ovale* (Weatherall and Abdalla, 1982). While 1 in 7 young red blood cells (RBCs) might be available for *P. vivax*, all RBC are equally susceptible to *P. falciparum* invasion (White et al., 1992).

Various possible mechanisms have been proposed to underlie the pathophysiology of SMA, but none of them has been properly studied due to the absence of appropriate models that could be used to dissect these mechanisms (Craig et al., 2012). A higher rate of erythrocyte destruction than the rate of erythrocyte production is thought to be a possible cause of SMA (Mackintosh et al., 2004). A proportion of Hb as high as 70% was thought to be digested by the

parasite prior to their removal (Abdalla and Pasvol, 2004). The rigid membrane of parasitized RBC is also thought to enhance their clearance by the reticulo-endothelial system (Poschl and Linderkamp, 1992, McCullough, 2014, Deng et al., 2015). The spleen is very effective in removing rigid RBC as well as sequestration of the parasitized cells. Furthermore, a substantial amount of RBC are also removed by intravascular haemolysis (Weatherall and Abdalla, 1982).

The deposition of parasite proteins on surfaces of both infected and uninfected cells as a result of unsuccessful parasite invasion attempts causes complement mediated lysis or uptake by macrophages (Sterkers et al., 2007), an immune mediated erythrocyte destruction (Molitor et al., 1991).

The presence of IgG and complement factors on the surface of erythrocytes as detected by means of the direct Coombs test (DAT) is associated with macrophage activation and erythrophagocytosis (Goka et al., 2001). A high rate of positive DAT is known to occur in anemic patients (Jeje et al., 1983b, Facer et al., 1979).

Immune mediated erythrocyte clearance is thought to account for continuous and partial erythrocyte removal after parasite clearance as sensitized erythrocytes are not immediately removed from the circulation (Facer et al., 1979).

Not all types of IgG on the infected erythrocyte surface can induce cell clearance. The presence of IgG1 and 3 is associated with higher haemolysis while IgG2 and 4 are associated with low-grade haemolysis (van der Meulen et al., 1980, Stratton et al., 1983), however, the amount of IgG was not reported. Another study showed that there is no correlation between the level of IgG on the erythrocyte surface and parasitaemia (Jeje et al., 1983a), hence the rate of immune mediated RBC destruction does not necessarily equate with the parasitic load and could partly explain the disparity between parasite density and anaemia.

Decreased RBC production results from marrow hypoplasia seen in acute

infections. Malaria induced dyserythropoiesis is a morphological observation, which in functional terms results in ineffective erythropoiesis (Zwadlo et al., 1987). Severely impaired bone marrow function is suspected in SMA cases with low parasitaemia; Children with SMA and low-grade parasitaemia are reported to have dyserythropoiesis and excessive growth of immature erythrocytes compared with those with acute malaria (Weatherall and Abdalla, 1982).

As parasites disappear from the blood either by therapy or host mechanisms, both haemoglobin level and bone marrow morphology return to normal (Abdalla, 1990), within 3 weeks in the convalescence phase (Phillips et al., 1986). This is an indication that malaria parasite clearance marks the onset of the recovery phase.

There has been controversy regarding the relationship between erythropoietin level and anaemia. Some groups reported that erythropoietin (EPO) level sometimes appears to be normal in SMA patients (Skorokhod et al., 2010, Mackintosh et al., 2004) while others have claimed that reduced erythropoietin level is associated with anaemia (Etzerodt et al., 2010, Van Gorp et al., 2010, Giha et al., 2010, Yu et al., 2011, Koda et al., 1998a). Other studies seem to resolve this controversy by reporting that normal levels of erythropoietin in the face of inadequate erythropoiesis could be caused by the accumulation of haemozoin in the bone marrow (Koda et al., 1998b, Rougemont et al., 1988) and the activities of cytokines (Font et al., 2001).

A product derived by lipid peroxidation catalyzed by haemozoin called 4-hydroxylnonenal (HNE) was reported to have an inhibitory role in erythropoiesis (Skorokhod et al., 2010). Haemozoin could induce cytokine imbalance through overproduction of IL-10 and IFN-α. Both IL-10 and IFN-α suppresses erythropoiesis via IL-12 (Font et al., 2001, Burte et al., 2013), a major cytokine that directly enhances erythropoiesis (Mohan and Stevenson, 1998). It is thought that

HNE directly influences erythroid progenitor cells and indirectly alters the production of inflammatory mediators such as TNF-α and Nitric Oxide (NO). Reduced NO might decrease the rate of parasite clearance and hence reduce erythropoiesis (Font et al., 2001).

1.44 Pathology of cerebral malaria and other clinical malaria syndromes

Cerebral Malaria (CM) is a clinical complication of malaria in humans with *P. falciparum* infection. It is characterized by convulsion and unarousable coma that persist for more than 30 minutes in the presence of asexual stage parasitaemia. The metric for the coma includes a Glasgow Coma scale <11 or Blantyre coma scale <3 (McGuire et al., 1994). The strict definition of CM allows it to be differentiated from other forms of malaria and encephalopathies and to allow the comparability of clinical investigation (Warrell et al., 1982, Taylor and Molyneux, 2015, McGuire et al., 1994, Polimeni and Prato, 2014).

CM occurs when the cerebral capillaries are blocked due to sequestration of parasite-infected RBC, RBC and residual bodies containing haemozoin (Mackintosh et al., 2004). A post-mortem study carried out on pediatric CM patients revealed different patterns of sequestration (Taylor and Molyneux, 2015), and a generalized multi-organ sequestration is a major feature of CM (Milner et al., 2015). Sequestration is thought to involve principally RBC infected with late trophozoites and schizonts, parasites in the second half of their intra-erythrocytic growth (Mackintosh et al., 2004).

Hypoxia is known to enhance parasite sequestration to the endothelial cell via parasite proteins such as PfEMP-1 binding to receptors such as ICAM-1, CD31 and CD36 (Turner, 1997, Idro et al., 2005, Brown et al., 1999, Storm and Craig, 2014). Sequestration is also enhanced through rosetting (the binding of infected to non-infected erythrocytes), auto-agglutination (the binding of infected erythrocytes

to each other) and platelet-mediated clumping of infected erythrocytes (Mishra and Newton, 2009). The sequestered parasites and RBC coupled with rigidified erythrocytes limit microvasculature flow and metabolic supply to the brain.

Severe haemolysis has also been reported in CM (Eisenhut, 2015, Poh et al., 2014), indicating that anaemia may be implicated in the pathology of CM. This anaemia accompanied by hypoglycemia causes seizure (Idro et al., 2005, Mishra and Newton, 2009).

Inflammation and permeability of the Blood Brain Barrier (BBB) are also implicated in CM. The permeability allows parasite molecules to get to the brain (Recuenco et al., 2014, Polimeni and Prato, 2014) and induce severe neurological dysfunction. Both CD8+ T cells (Polimeni and Prato, 2014) and Matrix Metalloproteinase (MMP) proteolytic enzymes are involved in the disruption of the inter-endothelial tight junction. MMP inhibitors are believed to prevent BBB dysfunction, reduce the inflammatory role of pro-inflammatory cytokines, reduce the high mortality rate in CM and ameliorate CM-associated neurological sequelae (Polimeni and Prato, 2014).

CM is thought to result in neurological impairment and sequelae, which may be transient or prolonged. Epilepsy was reported to be one adverse outcome of CM (Christensen and Eslick, 2015, Serghides et al., 2014). Sequelae of CM such as neurological impairments that are usually associated with intracranial pressure enhance mortality. Intracranial pressure is a fatal complication in CM and a major cause of death (Idro et al., 2005, Newton et al., 1997, John et al., 2008, Seydel et al., 2015). Increased brain volume was observed in some children with CM and it is strongly associated with neurological morbidity (Postels et al., 2014).

The cause of coma in children with CM is unclear; there is abrupt development of coma following seizure in children, however, in adults the development of coma is gradual and seldom follows seizure (Idro et al., 2005,

Mishra and Newton, 2009). A recent study reported that a high level of EPO is associated with prolonged coma and mortality in children of about 2 years-of-age and above and correlates with markers of endothelial and platelet activation and the presence of histidine-rich protein-2 (Shabani et al., 2015).

Cytokines also play a role in the pathogenesis of CM. High levels of Tumour Necrosis Factor-Alpha (TNF-α), IL-6 and IL-10 are associated with CM (Gimenez et al., 2003, Day et al., 1999). High levels of TNF-α, interleukin-1 (IL-1) and Transforming Growth Factor (TGF) were found localized in post-mortem analyses of brain tissue from CM patients (Mishra and Newton, 2009). On the other hand, anti-inflammatory cytokines such as IL-10 and IL-12 have been proposed to have regulatory and protective roles (Polimeni and Prato, 2014).

Studies show that bioavailability of NO might also improve disease outcome in children with CM (Xu et al., 2015, Trovoada Mde et al., 2014), while endothelial Protein C receptor was reported to be an early biomarker of poor prognosis and mortality in CM patients (Moussiliou et al., 2015). Furthermore, a high level of CRP might worsen the severity of CM and might play a role in human malaria; CRP deficient mice were reported to be more resistant to experimental CM (Szalai et al., 2014).

Other complications of malaria include respiratory distress that can occur at high parasitaemia or during convalescence following parasite clearance (McGuire et al., 1994). Malaria can also affect the blood sugar level because of the demand of the parasite for glucose, inducing a condition called hypoglycemia. This was observed in 8% of adults and 30% of children with CM (McGuire et al., 1994). Proteins that are involved in various aspects of glucose metabolism such as insulin-glucagon metabolism, and glycolytic enzymes are reported to be elevated in SMA and CM compared to UM groups (Bachmann et al., 2014). However, long treatment with quinine can stimulate insulin production leading to hypoglycemia.

Malaria induced hypoglycemia should be suspected when a patient with malaria shows altered behavior, impaired consciousness, tremor and breathlessness (McGuire et al., 1994).

Jaundice or hepatic dysfunction is more common in adults than children. While unconjugated hyper-bilirubinemia in jaundiced patients is an indication of extensive haemolysis of infected and non-infected erythrocytes, conjugated bilirubin is more likely to be the result of hepatocytic dysfunction (McGuire et al., 1994).

The passage of dark urine or black water fever occurs in acute malaria. It is thought to be a good prognosis in malaria. However, a few patients may develop acute renal failure following necrosis of the glomerulus tubules (Lange, 1992, Quaye, 2008, McGuire et al., 1994).

There is multi-organ damage in malaria because its pathology affects virtually all of the organs of the body (Krishnan and Karnad, 2003).

1.5 Extravascular and Intravascular haemolysis

Any removal of erythrocytes prior to their normal life span of 120 days is termed haemolysis. Intravascular haemolysis is characterized by the release of erythrocyte cellular components into the plasma. Extravascular haemolysis entails erythrocyte removal largely by the spleen without the release of erythrocyte components into the plasma (Dhaliwal et al., 2004).

Hemolysis may be asymptomatic until erythrocytosis no longer matches erythrocyte destruction. Clinical manifestations of haemolysis include either acute or chronic anaemia, reticulocytosis, dark urine and jaundice. The diagnosis is established by increased unconjugated bilirubin, elevated lactate dehydrogenase, decreased haptoglobin and peripheral blood smear and staining to detect reticulocytosis. The causes of haemolytic anaemia include immune-mediated

mechanisms, microangiopathy, hereditary RBC disorders and infections (Dhaliwal et al., 2004).

Immune-mediated haemolytic anaemia includes Autoimmune Hemolytic Anaemia (AIHA), transfusion induced or alloimmune haemolytic anaemia, and drug induced haemolytic anaemia. Antibodies directed against antigen on the erythrocyte induce haemolysis. The deposition of complement factor C3 on the surface of RBC leads to slow clearance by macrophages in the spleen and the liver while deposition of the membrane attack complex (C5b to C9) results in intravascular haemolysis (Dhaliwal et al., 2004, Engelfriet et al., 1992).

Drugs such as high doses of penicillin induce the expression of IgG antibody and causes extravascular haemolysis. Quinine could form a complex with IgM and could also induce haemolysis (Dhaliwal et al., 2004, Petz, 1993, Adner et al., 1968, Gottschall et al., 1991).

Endothelial damage within small blood vessels such as microangiopathy can cause an obstruction through the aggregation of platelets and fibrin. This obstruction within the vascular endothelium produces shearing force on the erythrocytes in circulation leading to intravascular haemolysis (Schrier, 2000, Maedel L and Sommer S, 1993). Disorders of erythrocyte enzymes such as glucose-6-phosphate dehydrogenase deficiency (G6PD) (Beutler and Luzzatto, 1999, Beutler, 1994, Steensma et al., 2001); the erythrocyte membrane such as hereditary spherocytosis (Konca et al., 2015, Comite Nacional de et al., 2015, Bolton-Maggs et al., 2012, Dhaliwal et al., 2004) and haemoglobin such as sickle cell anaemia and thalassaemia enhance haemolysis (Dhaliwal et al., 2004).

Malaria is both an inflammatory and haemolytic disease characterized by cycles of erythrocyte destruction. Cellular invasion by the parasite and its metabolic products enhance erythrocyte removal either intravascularly or extravascularly (Imrie et al., 2012). Intravascular haemolysis is an intrinsic feature

of severe malaria (Imrie et al., 2006) and it causes the release of free haem (fHb) into the plasma (Mendonca et al., 2012, Ferreira et al., 2008). Diverse and complex physiological pathways are normally deployed in mammals to control the oxidative effect of fHb when released from RBCs. There are specific proteins in circulation whose function is to ameliorate the toxic effect of fHb and its byproducts such as haem and iron. Control mechanisms start during erythropoiesis and extend through to haem and iron metabolism. Examples of a few proteins that drive these processes include Haptoglobin; CD163 receptors on macrophages; and Alpha Haemoglobin Stabilizing Protein (AHSP), an important protein implicated at an early stage of erythropoiesis (Imrie et al., 2012). AHSP functions as a chaperone, providing protection against oxidative damage to α-Hb subunits through specific binding (Kihm et al., 2002, Santiveri et al., 2004, Dickson et al., 2013, Mollan et al., 2013, Favero and Costa, 2011).

During intravascular haemolysis, fHb, haem and reactive oxygen or nitrogen species (ROS/RNS) are released into the plasma (Mendonca et al., 2012, Ferreira et al., 2008). There is further oxidation of fHb by actions of ROS and RNS to methaemoglobin ($MetFe^{3+}$), releasing more haem as well as exposing the haem prosthetic group. Further oxidation of both αβ subunit of the $MetFe^{3+}$ is possible yielding $MetFe^{4+}$ which is a transient intermediate molecule and a potent inducer of pro-inflammatory cytokine (Ferreira et al., 2008).

Oxidation of fHb transfers highly toxic free haem to the endothelial cell and to low-density lipoprotein (LDL) that can convert Lactate Dehydrogenase (LDH) to a cytotoxic oxidized product making the cell more prone to oxidative cellular injury (Balla et al., 2005). The iron moiety of fHb can also be oxidized by nitric oxide (NO) and this can further induce oxidative damage through mediators such as lipid and hydrogen peroxides (Han et al., 2002, Zukin et al., 2013, Fowkes et al., 2006). The fHb dimers that are released into the plasma during intravascular haemolysis

are thought to accumulate in malaria, particularly in SMA (Mendonca et al., 2012).

Both Hp and CD163 are involved in the metabolism of fHb dimer during haemolysis (Mendonca et al., 2012, Ferreira et al., 2008). Hp scavenges fHb and forms a complex with it (Quaye, 2008). This prevents the oxidation of fHb, nitric oxide damage, renal excretion of iron and any further oxidative damage (Sertorio et al., 2013). Once the complexes are internalized in macrophages, haem-oxygenase-1 (HO-1) enzymes degrade haem released from Hb into iron, bilirubin and carbon monoxide (CO) (Quaye, 2008, Mendonca et al., 2012, Sertorio et al., 2013, Schaer et al., 2006).

At low Hp plasma level, the plasma level of fHb increases following haemolysis (Delanghe et al., 1998c, Delanghe and Langlois, 2001). Hence, Hp is considered as a marker of haemolysis (Delanghe et al., 1998b, Gupta et al., 2011, Imrie et al., 2006, Kim et al., 2012a, Ko et al., 2013).

Similarly, LDH is known to be abundant in erythrocytes and its plasma level is elevated in the event of erythrocyte lysis. Elevated plasma level of LDH is also a surrogate marker of haemolysis in vascular pathophysiology (Ballas, 2013, Shah et al., 2014, Kato et al., 2006, Tabbara, 1992).

Hp has several biologic functions apart from its main role in fHb scavenging and its association with malaria has been widely reported (Langlois and Delanghe, 1996, Atkinson et al., 2014, Nyakeriga and Troye-Blomberg, 2013, Mendonca et al., 2012, Quaye, 2008, Delanghe et al., 1998c, Fowkes et al., 2006, Imrie et al., 2006).

1.6 The human Hp

Haptoglobin (Hp) is an acute phase protein. It's widely known as the scavenger of fHb, thereby preventing renal excretion of iron and oxidative damage as earlier mentioned. It is synthesized mainly in hepatocytes in response to

Interleukin 6 (IL-6), which is also synthesized in response to IL-1 and TNF-α (Quaye, 2008, Nielsen and Moestrup, 2009). IL-6 responsive regions on the Hp gene promoter include A-157, B-111 and C-61 (Quaye, 2008), while the A-165 to A-147 on Hp were found to be crucial for all hormone regulatory functions (Baumann et al., 1990). These cytokines and hormones were reported to promote *Hp* gene transcription (Quaye, 2008).

The plasma concentration of Hp increases with age and is measurable from 3 months to adulthood. Lower Hp level could be expected at 3-6 months of age. A concentration of 30-200 mg/dL is reached at about 20 years of age (Shinton et al., 1965). However, Hp level is dependent on location, Hp genotype, splenomegaly, α+-thalassaemia genotype, parasitaemia and age. There is a suspected negative correlation between Hp level and age at low parasite densities and a positive correlation at higher densities (Fowkes et al., 2006).

1.6.1 The structure of Hp protein and gene

The Hp gene is comprised of two alleles that are co-dominant; both Hp1 and Hp2 alleles are located on the long arm of chromosome 16 (Smithies, 1955, Smithies and Walker, 1956, Robson et al., 1969). The Hp1 allele has five exons and the Hp2 allele has seven (Fig. 1.3). The 5^{th} exon of Hp1 and the 7^{th} exons of Hp2 constitute the β-chain (Fig. 1.3 and 1.2). Both exon 3 and 4 of the Hp1 allele were duplicated to form the Hp2 allele such that the Hp2 allele is about 1.7kb longer (Yang et al., 1983, Wobeto et al., 2008b).

The Hp monomer is made up of two α and two β chains (Fig. 1.2). The α and β loci are linked such that a single mRNA generates the pro-Hp. The pro-Hp then undergoes proteolysis at position 161 (arginine) to yield the α and β-chains of Hp (Fig. 1.2). The α-chain represents 19% and the β-chain represents 81% of the total Hp molecule (Bier, 1967). Each β–chain is joined to an α-chain with a

disulphide bond (Fig. 1.2). The α-chains are also joined together with a disulphide bond (Fig. 1.2). There are also some disulphide bonds along each polypeptide within the α- and the β-chains (Kurosky et al., 1980, Raugei et al., 1983), which are suspected to be involved in the stabilization of the protein (Fig. 1.2).

The α-chains are classified as α1-chain and α2-chain and are present in the protein as either α1 or α2 or both (Koch et al., 2003). The *Hp*1 allele and *Hp*2 allele are so named due to the presence of α1 and α2 chains respectively. The Hp2-2 phenotype consists of two α2-chains (Fig. 1.2), Hp1-1 has two α1-chains and the Hp2-1 phenotype has one each of α1- and α2-chains.

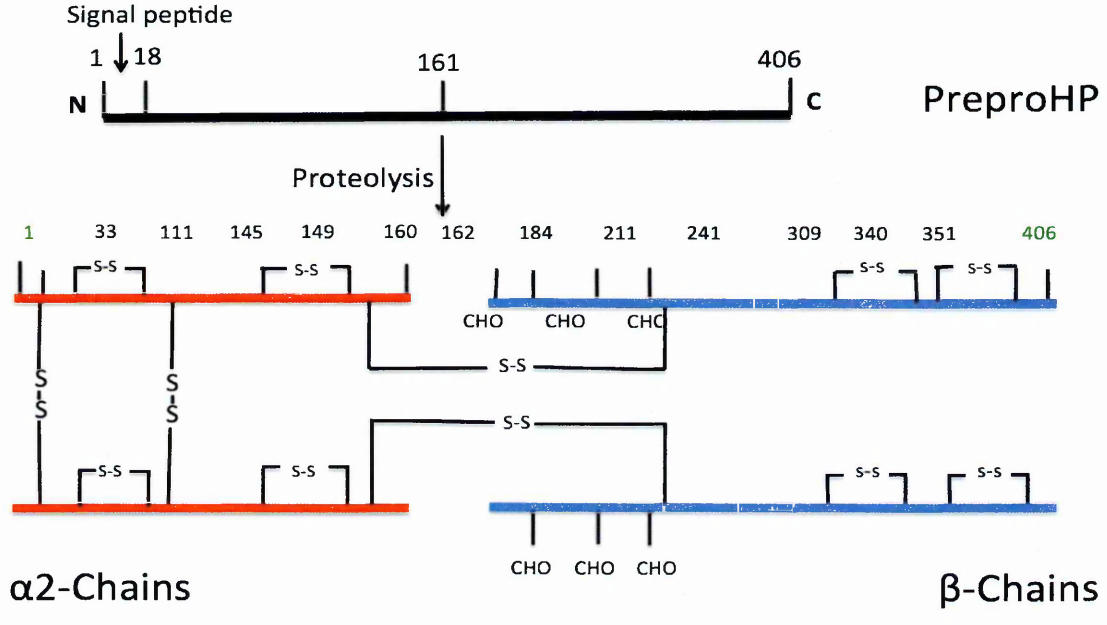

Figure 1.2 The structure of Hp2-2 protein dimer

The 406 amino acid precursor; PreproHp (black) has an N terminal signal peptide (residues 1 to 18), a proteolytic cleavage site at residue 161, and forms a dimer. Following removal of the signal peptide each protein in the dimer is cleaved into two fragments, the α2 chains (red) and the β chains (blue), which are held together by interchain disulphide bonds. The S-S represents disulphide bond. There are also intrachain disulphide bonds between cysteine residues as indicated, which are taught to stabilize the polypeptide. The numbers written in black on both the β and the α2 chains indicate other glycosylation (CHO) sites. The α1 chain of Hp1 is shorter than the α2 chain of Hp2 but the β chain is identical. Hp2-2 has two α2 chains, Hp1-1 has two α1 chains while Hp2-1 has one α1 and one α2 chain.

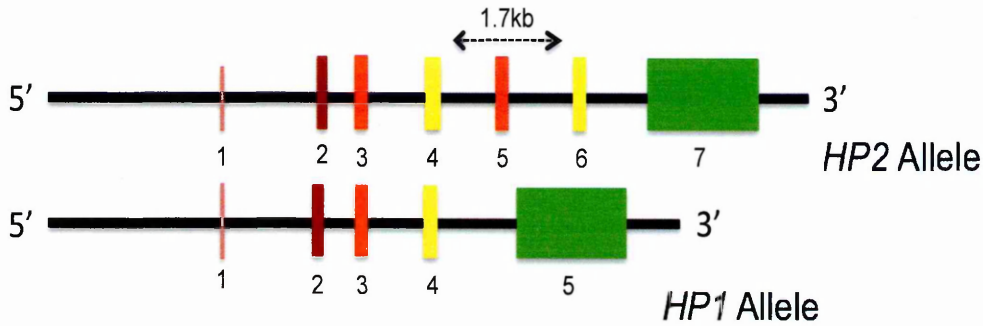

Figure 1.3 Diagram of Hp gene showing the difference between the Hp1 and the Hp2 allele

The *Hp2* and *Hp1*alleles are comprised of seven and five exons, respectively. The first six exons in the case of Hp2 and the first four exons in the case of Hp1 code for the α chain. Exons 5 and 6 of Hp2 are thought to have arisen by an internal duplication of exons 3 and 4, as indicated with the red and yellow colouring. The 3' exon (coloured green) codes for the β chain in both cases.

The α1-chain contains 83 amino acids while the α2-chain contains about 142 amino acids (Maeda, 1991, Wobeto et al., 2008b), so the α1 chain has a molecular weight (MW) of about 9kDa while the α2 MW is about 17kDa. Electrophoretic mobility on a gel after chemical reduction of Hp showed that the α-chains are sub-classified as slow (S) or Fast (F). The α1-chain is easily identified on a gel as α1S (alpha 1 slow) and α1F (alpha 1 fast). The difference between the α1S and α1F or α2S and α2F lies in the amino acid at positions 52 and 53 in the α_1 Chain and positions 111 and 112 in the α_2 Chain. Asparagine and glutamic acid at these positions gave rise to the "slow" form while aspartic acid and lysine at these positions gave rise to the "fast" forms (Connell et al., 1966, Koch et al., 2003).

The β-chain is heavier than the α-chain with a molecular weight of about 37kDa and is comprised of about 245 amino acids in all Hp types. The Hp β-chain is highly glycosylated. About 20% of the weight is carbohydrate present on the protein and sialic acid accounts for about 5% of this (Bier, 1967).

While the β-chain was not reported to be polymorphic (Wobeto et al., 2008a, Kurosky et al., 1980), the alpha chains are, resulting in 3 major

phenotypes, Hp1-1, 2-1 and 2-2 (Maeda et al., 1984, Langlois and Delanghe, 1996). The 'F and S' subtypes associated with both Hp1 and 2 alleles give rise to further phenotypes within the population. The possible allele combinations for Hp1-1 are Hp1F-1S, Hp1S-1S, and Hp1F-1F (Wobeto et al., 2008a). Possible sub-phenotypes associated with the Hp2-1 genotype include Hp2FS-1S, Hp2FS-1F, Hp2FF-1F, Hp2FF-1S, Hp2SS-1S and Hp2SS-1F while that of Hp2-2 include Hp2FS-2FS, Hp2SS-2FS, Hp2FF-2FS, Hp2FS-2SS and Hp2FS-2FF phenotypes (Koch et al., 2003).

Hp is known to bind free haemoglobin (fHb) in the ratio of 1:1 with a very high stability and affinity; $1X10^{-15}$ mol L^{-1} (Wobeto et al., 2008a). However, there are contradictory reports regarding the regions on both Hb and Hp that are involved in the binding. While some authors have suggested that it is the β-chain of Hp that binds the α-chain of Hb (Bier, 1967), other suggested that both the αβ chains of Hb and Hp are involved in the binding (Langlois and Delanghe, 1996); the β-chain of Hb binds to Hp at two specific sites, amino acids β11-25 and β131-146, while the α-chain binds Hp at position 121-127 (McCormick and Atassi, 1990).

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genotype include Hp2FS-1S, Hp2FS-1F, Hp2FF-1F, Hp2FF-1S, Hp2SS-1S and Hp2SS-1F while that of Hp2-2 include Hp2FS-2FS, Hp2SS-2FS, Hp2FF-2FS, Hp2FS-2SS and Hp2FS-2FF phenotypes (Koch et al., 2003).

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1.6.2 Ahaptoglobinaemia/hypohaptoglobinaemia

Hp is elevated in a wide variety of inflammatory diseases. However, its level decreases to an undetectable level in illness associated with haemolysis, (Blumberg et al., 1963). About 30% of the African population has an insufficient Hp level to permit phenotyping. This null/low Hp level in circulation is referred to as ahaptoglobinaemia/hypohaptoglobinaemia and is designated as Hp0 (Blumberg et al., 1963).

Studies on hypohaptoglobinaemia in malaria have provided conflicting views regarding its aetiology in childhood malaria: on one hand, a low level of plasma Hp in malaria was proposed to have a genetic cause, while on the other hand it was attributed to the disease causing intravascular haemolysis (Delanghe et al., 1998b).

There is slight variation in the frequency of hypohaptoglobinaemia in different populations within the same country (Constans et al., 1981). Its average

frequency in Africa was reported to be 40% (Teye et al., 2004), whilst that of ahaptoglobinaemia was reported to be 1 in 1,000 Caucasians. The average frequency of hypohaptoglobinaemia in Nigeria and Cameroon was 30% and that of central part of Nigeria alone was reported to be within 65-76%, however, the frequency in persons under constant anti-malarial drugs was reported to be 30% lower (Allison et al., 1958, Giblett, 1959, Langlois and Delanghe, 1996). The frequency of hypohaptoglobinaemia in North Americans of African descent was reported as 2-4% and is generally lower in Africans not residing in malaria holoendemic regions (Giblett, 1959, Langlois and Delanghe, 1996), As a result, ahaptoglobinaemia was reported to be a consequence of malaria infection (Boreham et al., 1979) and it's acquired in areas where malaria is endemic and untreated (Boreham et al., 1979). In spite of this, it is still not clear whether a/hypohaptoglobinaemia is a consequence of, or a predisposing factor to malaria.

A 5.2 kilobase (kb) deletion of the *Hp* gene, spanning from upstream of exon 1 to between 52 and 53 base pairs (bps) upstream of exon 5 of the adjacent haptoglobin-related (HPR) gene, was reported to be associated with null Hp level. The frequency of this deletion was 0.051, 0.031, and 0.060 in Koreans, Japanese, and Chinese respectively. However, no such deletion was present in 101 Africans and 100 Europeans of African decent (Koda et al., 2000).

The homozygous genotype deletion results in complete absence of serum Hp; heterozygous deletion such as the deletion of only the *Hp1* allele (*Hp2/Hp1*^{del} genotype) is associated with extremely low Hp level and the deletion of *Hp2* allele (*Hp2*^{del}*/Hp1* genotype) is associated with an Hp level that is approximately 50% of that observed in normal genotype. The frequencies of these heterozygous deletions in Japanese, Chinese and Korean populations is within 15-30% (Wobeto et al., 2008a). No gene deletion or re-arrangement was found in a Vanuatu (Hill et al., 1987); southern and western Asian; European; and African (Gambian)

populations (Soejima et al., 2007).

Three previously known base substitutions (A-55G, A-61C and T-104A) together with three new ones (C-101G, T-191G and C-242T) within the 5' flanking region of the Hp gene were identified. The A-61C base substitution was reported to significantly decrease transcriptional activity and was associated strongly with the Hp2 allele and ahaptoglobinaemia. The C-101G substitution was found to be similar in transcriptional activity to the wild type and was associated with Hp1S allele and hypohaptoglobinaemia (Teye et al., 2003).

A heterozygous T→C substitution at codon 247 in the β-chain of the Hp2 allele in the Ghanaian population caused reduced expression of the protein when transfected into COS7 cells. This base substitution resulted in a missense change of the non-polar amino acid isoleucine to the polar amino acid threonine. Aside from the A–61C mutation, which leads to low *Hp* expression, this codon substitution could be another etiologic cause of ahaptoglobinaemia, possibly in African population (Teye et al., 2004).

1.6.3 Geographical Frequency distribution of Hp

Hp phenotypes are known to show marked frequency variation with geographical location and race. There is a general increase in the Hp2 allele frequency among Korean, Indian, Afghanistani, Australian and European populations. The frequency of the *Hp*1 allele is higher in African populations. West Africa has the highest frequency of the *Hp*1 allele. The frequency of Hp1 and Hp2 alleles in Southern Africa is approximately the same but the Bushmen in South Africa and Botswana have a lower *Hp*1 allele frequency of about 20-30% compared to the rest of the population. The *Hp*1 allele frequency in regions representing the North Eastern borders of Africa decreases, ranging from 30% in Djibouti, 40% in Ethiopia and 21% in Egypt. The northern part of Algeria has a

lower *Hp*1 frequency than the southern part (Teye et al., 2006, Elagib et al., 1998, Mavondo et al., 2012, Carter and Worwood, 2007, Constans et al., 1981, Buettner-Janusch et al., 1973, Hashem et al., 1966, Giblett et al., 1966, Buettner-Janusch and Buettner-Janusch, 1964, Pllitzer, 1963, Goldschmidt et al., 1962). A wide range in the frequency of the *Hp*1 allele exists in India, China, Indonesia and Russia as well as in Australia. The aboriginal Australian population have the lowest frequency of the *Hp*1 allele (Wobeto et al., 2008a, Pllitzer, 1963, Goldschmidt et al., 1962).

Unlike in Africa, the frequency of the *Hp*1 allele in Europe is fairly stable, around 35-40%. The Sami people in the north region of Finland, Sweden and Norway have an *Hp*1 allele frequency of 31-32%. However, wide variations in Hp allele frequencies exist from the southern to the northern part of Italy (Goldschmidt et al., 1962, Carter and Worwood, 2007). The *Hp*1 allele frequency in the Middle East and Asia is fairly stable between 20-35%. Jewish people have a very stable frequency of 30-33% across the globe.

Diverse ethnic groups characterize the American population and therefore the *Hp*1 allele in Americans was considered based on race. The black American has the highest frequency of *Hp*1, although it is about the same as in those of Hispanic origin. The frequency of the *Hp1* allele among native Americans in Canada and the USA ranges from 41-54%. The indigenous peoples in South America have a higher percentage of *Hp*1 allele that is about the same as those in West Africa (Wobeto et al., 2008a, Pllitzer, 1963, Goldschmidt et al., 1962, Langlois and Delanghe, 1996).

The frequency of the various subtypes is not usually ascertained; however, a study in England indicated that the frequency of Hp2FS is the highest while Hp2FF is the lowest. The Hp1S is about 24-28%, Hp1F is about 11-15%, Hp2FS is about 54-63%, Hp2SS is about 5-6% and Hp2FF is about 0-1%. An extreme

variation exists in the frequency of the Hp1F allele, particularly in India and Nigeria and there is a gradually increasing gradient in the frequency of Hp2FS, particularly from the West to the East. (Mastana et al., 1994, Mastana and Fisher, 1994).

1.6.4 Biological significance of Hp

Hp is involved in a number of biological actions such as the immune response, regulatory effects on nitric oxide (NO), prostaglandins, angiogenesis, antibody-like function, antibacterial-like function and antioxidant functions (Kasvosve et al., 2010, Wobeto et al., 2008a).

Table 1.1 Summary of the biologic function of Hp

S/N	BIOLOGIC FUNCTION OF Hp	
1.	Protection against oxidative stress	Hp protects against oxidative stress by protecting the iron moiety of haem from free radicals such as superoxide and hydroxides by binding to fHb.
2.	Inhibition of nitric oxides	Nitric oxide (NO), originally referred to as endothelium-derived relaxing factor (EDRF). Free Hb and Hp bound to free Hb inactivate NO/EDRF, whereas Hp does not.
3.	Angiogenic function	Hp stimulates tissue repair and compensates for ischemia by promoting the growth of collateral vessels.
4.	Immunomodulation	Hp phenotypes are associated with response to vaccination. Hp2-2 produces higher antibody titre

		than Hp1-1 and Hp2-1 individuals. The complex formed between Hp and fHb produces anti-inflammatory cytokines on binding to CD163. Hp also binds with neutrophils as well as B-cell receptors via CD22.

1.6.4.1. Hp protection against oxidative stress

Oxidative stress has been suggested to be involved in pathophysiology of malaria. The imbalance between oxidant and antioxidant has been reported to cause severe damage (Tiyong Ifoue et al., 2009, Araujo et al., 2008).

Oxidative stress is when the pro-oxidant exceeds the antioxidant (Araujo et al., 2008). Hp protects against free radicals such as superoxide and hydroxyl by binding to fHb thereby shielding the iron moiety of haem within the porphyrin ring of fHb. The presence of fHb causes the accumulation of these free radicals, particularly hydroxyl radicals that are formed between biological hydroperoxides and Fe_2+ in the haem moiety ($H_2O_2 + Fe_2+ = 2OH- + Fe_3+$). These Free radicals cause damage by lipid peroxidation, for example, haem iron catalyzes the oxidation of low-density lipoprotein (LDL), making it capable of damaging endothelial cells (Balla et al., 1991) The Hb binding capacity (HBC) of Hp as well as the affinity of the Hp-Hb complex for CD163 is dependent on the Hp phenotype.

The HBC measures the capacity of an Hp phenotype to protect against haemolysis and oxidative stress. The HBC of Hp is ranked in the order Hp1-1>Hp2-1>Hp2-2, while the binding of their respective complex to CD163 is ranged in the order of Hp2-2>Hp2-1>Hp1-1 (Kasvosve et al., 2010, Quaye, 2008). This is because though all Hp phenotypes have enough binding capacity for Hb, their ability to scavenge fHb is associated with the protein's size, Thus, Hp2-2 being the largest moves fHb to the extravascular space more slowly, allowing

greater oxidative stress due to the presence of more fHb in circulation. Hp1-1 on the other hand is more protective against oxidative damage (Koda et al., 1998b). Higher circulatory levels of fHb bind and scavenge NO. Therefore, Hp2-2, which is thought to leave more fHb in the circulation is associated with high plasma haem, increased NO consumption and low plasma nitrite compared to Hp1-1, which on the other hand is thought to have a protective role in this regard (Sertorio et al., 2013).

The addition of Hp to RBC during storage prior to transfusion helps to improve the quality of stored RBC by preventing loss of NO through scavenging of fHb (Wang et al., 2014c). The movement of Hp/fHb complex into the macrophage is not applicable in this regard, as Hp is only required to bind fHb and prevent it from depleting the NO. In this case, Hp1-1 with the highest affinity to fHb will best maintain the quality and NO content of stored RBC.

1.6.4.2 Hp effect on endothelial vasodilation

Many cells including macrophages produce NO through the activities of appropriate cytokines. The biologic function of NO is a double-edged sword and complicated. Large amounts of NO could act against parasites and microorganisms but they are also cytotoxic. On the other hand, small amounts of NO are involved in maintenance of vascular tone, affecting the contractile state of the smooth muscle cells found in the vessel wall. Decreased tone means larger diameter of the vessel, decreased resistance and more supply to the appropriate organ. Increased tone implies smaller diameter and more resistance to the flow. NO is also described as an Endothelial Derived Relaxing Factor (EDRF) (Langlois and Delanghe, 1996, Edwards et al., 1986) and plasma fHb inhibits endothelial vasodilation through direct interaction with EDRF. Hp and fHb have opposing effects on the endothelial vessel; while both fHb and Hp/fHb complex prevents its

dilation, Hp prevent relaxation; decrease in tone (Langlois and Delanghe, 1996, Edwards et al., 1986).

1.6.4.3 Angiogenic effect of haptoglobin

Angiogenesis is an important process in recovery from inflammation and a number of pathological conditions. Purified Hp has been reported to stimulate angiogenesis in a dose dependent manner in patients with vasculitis. Increased Hp during inflammation and ischemia plays an important role in tissue repair and vessel development. Anti-haptoglobin antibody inhibits angiogenesis. Furthermore, a correlation between angiogenic activity, disease and serum level of Hp have been reported (Cid et al., 1993). The human *Hp2* gene transfected into Endothelial Progenitor cells (EPC) induced the expression of pro-angiogenic factor genes.

While the Hp2 allele is important in angiogenesis, the Hp1 allele suppresses angiogenesis (Park et al., 2009). Other reports indicated that Hp2-2 has more angiogenic function than both Hp2-1 and Hp1-1 (Langlois and Delanghe, 1996). The levels of serum angiogenic factors are higher in Hp2-2 compared to both Hp2-1 and Hp1-1 (Weissgerber et al., 2012).

1.6.4.4 Immuno-regulatory function of Hp

The role of inflammatory mediators in directing host immune functions in relation to infectious and non-infectious disease is becoming an intensively studied process. Hp is a glycoprotein that regulates the immune response (Mandato et al., 2012, Abdullah et al., 2009), ranging from immunosuppression (Oh et al., 1990b), to inhibition of lymphocyte reactivity or inducing the biosynthesis of IgG (Lange, 1992) and Hp2-2 has a stronger effect than the two other Hp types in this regard. The tight correlation between inflammation and induction of Hp expression by cytokines further led to the notion that Hp may play a regulatory role in immune

function.

Aside from the proposed anti-inflammatory activity of plasma Hp during the course of an acute phase reaction (Huntoon et al., 2008), Hp also inhibits prostaglandin synthesis and protects against harmful oxidation processes (Lange, 1992).

CD22 is a receptor of resting mature B-cells that recognizes α2-6 sialylated glycoprotein on Hp and other sialylated proteins in the plasma (Powell et al., 1993, Hanasaki et al., 1995). Sialylated Hp could therefore bind to B-cells through CD22 and modulate lymphocyte antigen recognition (Law et al., 1994, Stamenkovic and Seed, 1990). Hp affects the proliferation and cytokine production of stimulated T cells and B cells (Galicia et al., 2009). For example, 2mg/ml Hp concentration inhibited B-cell proliferation while 0.5-1mg/ml enhanced it (Baseler and Burrell, 1983).

Hp suppresses secretion of TNF-α, IL-10, and IL-12p70 from macrophages (Galicia et al., 2009). This regulatory function of Hp on cytokine secretion could also be linked to its ability to bind human mast cells via interaction with Macrophage-1 (MAC-1) or CD11b/CD18 (El-Ghmati et al., 2002). This binding via MAC-1 present on neutrophils, NK and mononuclear phagocytes like macrophages (Galicia et al., 2009) could modulate both MAC-1 and mast cell functions (El-Ghmati et al., 2002, El Ghmati et al., 1996). Human Mast cells are known to induce production of pro-inflammatory cytokines, histamines, growth factors and proteases (Jung et al., 2013) and also play a role in allergic reactions such as rhinitis and urticaria (El-Ghmati et al., 2002).

Hp may modulate both innate and adaptive immune responses; it is reported to have two binding sites on neutrophils and inhibits several of their functions (Galicia et al., 2009), including neutrophil respiratory burst activity (Oh et al., 1990b). Hp as a product of tumour cells interferes with innate and adaptive

immune function and its elimination in cancer patients may serve as an immunotherapy (Oh et al., 1990a).

The Hp/fHb complex is also known to modulate the immune system; binding of Hp/fHb complex to CD163 could induce Th-1 pro-inflammatory cytokine or Th-2 anti-inflammatory cytokine (Van Gorp et al., 2010). Hp1-1 complexed with fHb and bound to CD163 stimulated the secretion of IL-6, IL-10 and TNF-α (Guetta et al., 2007). The binding of Hp/fHb complex to CD163 activates an anti-inflammatory response via IL-10, which in turn increases the expression of CD163 and further enhances Hp/fHb scavenging via a positive feedback mechanism (Van den Heuvel et al., 1999, Van Gorp et al., 2010). While fHb/Hp1 complex bound to CD163 receptor induces the secretion of anti-inflammatory cytokines, fHb/Hp2 bound to CD163 is not known to induce anti-inflammatory cytokines (Guetta et al., 2007, Strauss and Levy, 2008). The modulation of the balance between pro/anti-inflammatory cytokine by Hp genotypes exposed to fHb explains partly the inter-individual response to haemolysis associated inflammatory disease (Guetta et al., 2007).

Hp interacts with both resting and activated CD4+ and CD8+ T-cells, leading to the suppression of T-cell activation. Hp inhibits the production of Th-2 cytokines; both IFN-γ and IL-2 production are slightly inhibited at high Hp level. Hp deficient mice could not produce IFN-γ. Hp therefore is an acute phase protein that could play an immunoregulatory role particularly in the balance between Th-1 and Th-2 cytokines (Arredouani et al., 2003).

Hp isoforms are associated with antibody titre response to *Salmonella typhi* vaccine (Nevo and Sutton, 1968). Individual with Hp2-2 are reported to have the highest anti-type O titre after immunization with *S. typhii* vaccine (Nevo and Sutton, 1968). However, Hp2-2 individuals produced lower antibody titres following influenza and hepatitis B vaccination but stronger antibody responses with tetanus

(Kasvosve et al., 2010, Delanghe et al., 1998c).

Hp phenotype is related to the titre of agglutination against streptococci carrying a T4-antigen (Prokop et al., 1979, Gunther et al., 1979). Higher agglutination titre was observed in pregnant Hp2-2 women. The agglutination properties of both Hp2-2 and Hp2-1 were inhibited by addition of Hp1-1 anti-sera. Also, absorption of Hp by affinity chromatography decreased the agglutination titre for T4 streptococci (Prokop et al., 1979, Gunther et al., 1979). The agglutination might have been mediated via a lectin-like binding since both Hp2-2 and 2-1 do not have the variable antigen-binding sites that characterize an IgG and more so that the agglutinations do not induce complement activity (Langlois and Delanghe, 1996).

The association between Hp and IgA in allergic rhinitis has also been reported (Khazaei et al., 2012).

1.6.5 Haptoglobin phenotypes in infectious and non-infectious diseases

As earlier mentioned, Hp is an important immunomodulator; Hp suppresses T-cell proliferation and cytokine release. Th2 cytokines are more suppressed than Th1 cytokines and therefore the concentration of Hp is important in determining the ratio of both cytokines which are important in disease susceptibility (Kasvosve et al., 2010).

The binding between fHb and Hp makes iron unavailable for microbial growth, and therefore it's protective against bacterial and non-bacterial infections that requires iron such as HIV (Kasvosve et al., 2010), and particularly HIV-1 (Quaye et al., 2000a).

Association between Hp phenotypes and various infections have been reported. Individuals with Hp1-1 are prone to *Streptococcus pyogenes* infection. Hp1-1 is also associated with Epstein bar virus, Hepatitis B and C and malaria

infection. On the other hand, Hp2-2 has a protective role in *Streptococcus pyogenes* infection but it's associated with higher risk of mortality in tuberculosis and HIV. Hp2-2 is prevalent in individuals with trypanosomiasis while Hp2-1 is prevalent in those with legionella disease.

1.6.5.1 Haptoglobin polymorphism and malaria

Hp1-1 is reported to be associated with susceptibility to severe malaria in Africa (Elagib et al., 1998, Quaye et al., 2000b) in spite of its higher frequency in the region. This frequency of the phenotype in the region is probably sustained by conferring protection from other diseases. The Hp2 is thought to have spread under the influence of a strong selection pressure but its unclear whether it arose as a result of protection from malaria or another disease. Children with Hp2-2 are reported to have 30-50% reduction in clinical malaria episodes while children with Hp1-1 have a reduced rate of non-malarial fever but a high rate of malarial fever (Atkinson et al., 2007).

There are conflicting reports in the literature on the role of Hp polymorphism in malaria (Kasvosve et al., 2010); while some authors reported that Hp1-1 is associated with susceptibility (Elagib et al., 1998, Quaye et al., 2000b, Minang et al., 2004, Atkinson et al., 2007), others did not find any association (Idro et al., 2008, Aucan et al., 2002, Bienzle et al., 2005). Some authors thought that the contradiction is due to an A-61C (rs5471) mutation found in the promoter region of the *Hp* gene. It was reported that this SNP is associated with low circulatory Hp level and protects from malaria as much as the sickle cell trait (Cox et al., 2007), and was reported to be of higher frequency in the Hp2 than the Hp1 allele (Cox et al., 2007). It was reported that the low plasma Hp level associated with the A-61C could be reversed following malaria treatment (Cox et al., 2008, Quaye, 2008), however, it is very unlikely to reverse a genetic effect as was reported. Low Hp

levels due to a genetic effect could not be reversed; hence, the low Hp levels observed with A-61C could be haemolysis or disease associated rather than genetic.

We propose that while low Hp levels as a consequence of malaria could be reversed, low levels due to a genetic effect cannot be reversed. A study has reported that Hp levels recovered in 75% of the study population following anti-malarial treatment but not in the remaining 25% (Joshi et al., 2002). The etiologic cause in those 25% was likely to be genetic. Defining the aetiology of Hp is very important to understand its effect in the pathophysiology of malaria.

1.6.5.2 Hp association with non-infectious diseases;

Cancer

Hp is a serum protein biomarker for Non-small Cell Lung Cancer (NCLC) (Ayyub et al., 2015, Wang et al., 2014a) and is important in evaluating the histological type of lung cancer (Ayyub et al., 2015, Hoagland et al., 2007). A high level of Hp is associated with NCLC such as squamous cell carcinoma and adenocarcinoma compared to controls (Ayyub et al., 2015, Abdullah et al., 2009). Levels in squamous carcinoma are higher than in adenocarcinoma (Wang et al., 2014a).

High levels of Hp were reported in small cell lung cancer (Shah et al., 2010, Everaert et al., 1998b). A more detailed study identified Hp α-chain as the specific biomarker for NCLC (Park et al., 2013) and ovarian cancer (Delanghe et al., 1998a). Hp β-chain is reported to be a non-specific biomarker for early detection of prostate cancer (Saito et al., 2008). The association of Hp polymorphism and development of cancer depends on the type of cancer. A high frequency of Hp1-1 and family history have been reported to be important in breast cancer (Awadallah

and Atoum, 2004, Wobeto et al., 2008a). Serum level of Hp increases with the stages of lung cancer (Fedorovych et al., 1995) and Triple Negative Breast Cancer (TNBC). Higher Hp level is associated with grade 3 compared to grade 2 TNBC in young women (Tabassum et al., 2012).

A higher frequency of the Hp1 allele, particularly in the heterozygous Hp2-1 is associated with ovarian cancer, oesophageal cancer, and leukaemia. The Hp2 allele, particularly in the homozygous Hp2-2 phenotype is associated with lung, oesophageal and gastric cancer. Hp2-2 patients have a better prognosis for epithelial ovarian cancer and also elicit better immune responses than those with Hp1-1 and 2-1 phenotypes (Mandato et al., 2012, Zhao et al., 2007).

A reduced risk of cervical cancer is associated with Hp2-2 (Quaye et al., 2009) while Hp polymorphism is not associated with the development of prostate cancer and does not determine the disease outcome (Mavondo et al., 2012). However, some reported the use of fucosylated Hp as a novel prognostic biomarker for prostate cancer (Fujita et al., 2014, Fujimura et al., 2008), distant and liver metastasis in colorectal cancer (Takeda et al., 2012, Sun et al., 2012), colon cancer (Park et al., 2012), pancreatic cancer (Lin et al., 2011, Miyoshi et al., 2010, Nakano et al., 2008, Miyoshi and Nakano, 2008, Okuyama et al., 2006) and in cancer treatment using a peptide vaccine (Pang et al., 2013). The level of fucosylated Hp is high during the disease and low on recovery (Thompson et al., 1992b, Thompson et al., 1992a, Thompson et al., 1991).

Glycosylated forms of Hp are also a marker for monitoring disease progression in a number of cancers including lung cancer (Tsai et al., 2011, Shah et al., 2010, Bresalier et al., 2004, Thompson et al., 1992a); The Hp/fHb complex has also been shown to be cytotoxic to human hepatocellular carcinoma cells by inducing apoptosis and DNA fragmentation (Kim et al., 1995).

Diabetes

Hp1-1 may delay the onset of diabetes while Hp2-2 poses a microvascular risk (Goldenberg-Cohen et al., 2011). Diabetic patients with Hp2-2 are under increased oxidative stress as compared with those expressing Hp1-1 or Hp2-1 (Awadallah et al., 2013).

Individuals with Hp2-2 develop more complications or sequelae such as cardiovascular complications than those with Hp1-1 while an intermediate risk is associated with Hp2-1 (Wobeto et al., 2008a). Hp1-1 protects diabetic patients from narrowing of blood vessels (restenosis) following the implantation of a coronary stent and it is associated with a smaller size of infarct in myocardial infarction (Wobeto et al., 2008a). The Hp1-1 genotype is associated with lower coronary artery disease risk in diabetes, protects from diabetes-associated nephropathy (Wobeto et al., 2008a) and an increased risk of stroke incidence in type-1 diabetes (Costacou et al., 2014).

Diabetics with Hp2-2 are more predisposed to developing microvascular complication and retinopathy. Type-2 diabetes individuals with Hp2-2 develop more cardiovascular complications than those with Hp1-1 while an intermediate risk is associated with Hp2-1 (Wobeto et al., 2008a, Adams et al., 2013, Vardi and Levy, 2012, Vardi et al., 2012, Shi et al., 2012, Kurosky et al., 1980, Ryndel et al., 2010).

The cardiovascular complication associated with Hp2-2 is due to impaired antioxidant function. Hence, vitamin E supplementation in Hp2-2 diabetics is clinically effective and can prolong life (Blum et al., 2010, Nakhoul et al., 2009).

Hp2-2 also predisposes to atherosclerosis (Wobeto et al., 2008a) and it is associated with lower arterial elasticity, vascular resistance, microvascular complications, poor endothelial function in diabetics compared to controls (Dayan et al., 2009), refractory hypertension in type-2 diabetes mellitus (Wobeto et al.,

2011), and retinopathy (Wobeto et al., 2008a).

Hp modulates glycemic control and cognitive function in type-2 diabetes. Hp1-1 patients with type-2 diabetes and poor glycemic control have impaired cognitive ability (Guerrero-Berroa et al., 2015). People with type-1 diabetes develop renal disease despite good metabolic control. A greater risk of renal disease is associated with Hp2-2 (Orchard et al., 2013).

Cardiovascular disease and atherosclerosis

Hp2-2 is a risk factor for coronary atherosclerosis and peripheral arterial occlusive disease (Delanghe et al., 1999). Hp2-2 patients are 5-6 times more likely to suffer from subclinical atherosclerosis than Hp1-1 and Hp2-1 patients respectively, (Ragab et al., 2014). Furthermore, Hp is a significant predictor of premature atherosclerosis in children with β-thalassaemia major.

Hp is an independent risk factor for the development of cardiovascular disease (CVD) particularly when there is an underlying diabetes (Levy et al., 2002, Levy, 2004, Blum et al., 2008, Costacou and Levy, 2012, Adams et al., 2013). It is also a risk factor for obstructive sleep apnea (Lavie et al., 2003) and chronic kidney disease (CKD) (Strandhave et al., 2013); Hp2-2 negatively correlates with decreased heart rate variability and high CRP protein in patients with CKD (Strandhave et al., 2013).

1.6.6. Other inflammatory and haemolysis associated defence proteins

1.6.6.1 CD163

Macrophages and their progenitor cells, the monocytes are part of the immune system with a variety of functions displayed via the different molecules expressed at their surface. They are distinguished by such signals, secretory

products and expressed biomarkers. One such molecule is the scavenger receptor CD163 that was used to detect a specific macrophage phenotype associated with the healing phase of the inflammatory process (Zwadlo et al., 1987, Etzerodt et al., 2010, Van Gorp et al., 2010). The expression of CD163 is restricted to cells of the monocyte/macrophage lineage. CD163 is present on resident tissue macrophages such as red pulp macrophages in the spleen, Kupffer cells in the liver, and interstitial and alveolar macrophages in the lung (Van den Heuvel et al., 1999, Van Gorp et al., 2010). CD163 is a 130kDa membrane protein that is extensively glycosylated; its size could be significantly reduced by treatment with glycosidase (Van Gorp et al., 2010). It has a cytoplasmic tail, and trans-membrane and extracellular domains. The phenotypes of CD163 are differentiated based on the length of its cytoplasmic tail. The extracellular domain consists of about nine cysteine-rich scavenger receptors (CRSR) motifs of about 110 amino acid residues (Fig. 1.4) and is similar to other scavenger receptor superfamily proteins (Etzerodt and Moestrup, 2013, Graversen et al., 2002). CD163 plays a role in the resolution of inflammation and is more expressed at the site of chronic inflammation (Sulahian et al., 2004, Moestrup and Moller, 2004). However, its principal function is to serve as a receptor for scavenging fHb/Hp complex into the macrophages (Schaer et al., 2006).

The binding of fHb/Hp complex to CD163 is dependent on physiological calcium concentration; there is no binding at low calcium concentrations (Madsen et al., 2004). As mentioned, the internalized fHb is degraded to iron and bilirubin (Fig. 1.4), particularly in liver macrophages thereby curbing the toxic effect of fHb (Sulahian et al., 2004). Furthermore, the fHb/Hp-CD163 pathway only functions when the plasma Hp level is high in relation to Hb. In the event of low/null Hp levels, a direct uptake of fHb by CD163 is possible. Uptake of fHb without CD163 is also possible (Schaer et al., 2007).

Following the internalization of fHb/Hp by CD163, the intact CD163 is shed from the surface of the macrophage as soluble CD163; sCD163 (Sulahian et al., 2004, Adly et al., 2015), through the actions of inflammatory mediators (Fig. 1.4) and both TNF-α Converting Enzymes and a Disintegrin Metalloproteinase-17 (TACE/ADAM17) that are thought to be responsible for the cleavage (Thomsen et al., 2013) by an as yet unknown mechanism. The binding of Hp/fHb complex to CD163 produces anti-inflammatory cytokines such as IL-10 (Perdijk et al., 2013). Secondly, haem metabolites such as bilirubin resulting from the degradation of scavenged fHb also have potent anti-inflammatory effects (Fig. 1.4).

Evidence from mass spectrometry analysis showed more than 94% identity between both sCD163 and the CD163 extracellular domain (Moller et al., 2010, Van Gorp et al., 2010). Hence, sCD163 could also bind fHb/Hp complex and prevent the toxic effect of fHb. However, its scavenging function is limited because its circulatory level is approximately 1000-fold lower compared to Hp (Madsen et al., 2004).

Both sCD163 and CD163 are also receptors for erythroblast adhesion, Tumour necrosis factor-like weak (TWEAK) inducer of apoptosis and some pathogens. These interactions could result in receptor-mediated endocytosis and are capable of triggering the production of signaling molecules and that makes them function as immunomodulators and potential targets for cell-directed immune therapy (Van Gorp et al., 2010). The sCD163 can also act like an anti-inflammatory, exhibiting cytokine-like function by decreasing T-lymphocyte activation and proliferation (Frings et al., 2002, Davis and Zarev, 2005).

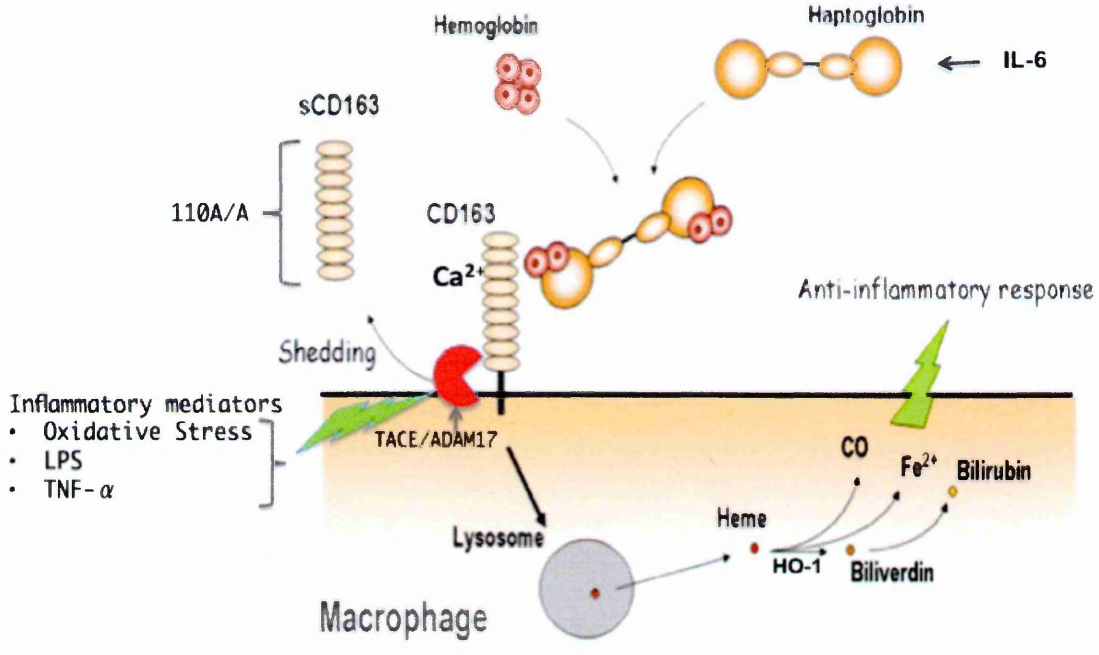


Figure 1.4. fHb-Hp Scavenger-Receptor Mechanism

IL-6 induces the expression of Hp, which binds to fHb in the plasma. Both Hp and fHb forms a complex that binds to CD163. This binding is mediated by the presence of physiological calcium. fHb internalized into the macrophage is degraded in the lysosomes, the haem moiety is released to be degraded by HO-1 to biliverdin that is then converted to bilirubin; Fe^{2+}; and CO. The binding of Hp/fHb complex to CD163 activates TACE/ADAM17 through inflammatory mediators to cleave CD163 as sCD163. sCD163 has 110 amino acids and is very related to CD163. The diagram was adapted with modification from Moestrup and Moller (Moestrup and Moller, 2004) and Etzerodt and Moestrup (Etzerodt and Moestrup, 2013)

In a clinical setting, sCD163 is of significance because it is being used as a biomarker of macrophage activation during inflammation (Adly et al., 2015).

Clearance of sCD163 could be through its binding with TWEAK, a secreted cytokine belonging to the TNF-α superfamily. TWEAK is also capable of stopping the biological actions of sCD163 (Etzerodt and Moestrup, 2013).

1.6.6.2 *Hemopexin*

HPx is a plasma glycoprotein that is produced primarily in the liver and other secondary sites such as the nervous system, skeletal muscle, and kidney. It is regulated during haemolysis, haem overload and inflammatory events and has been considered as an acute phase protein (Fiorito et al., 2013, Tolosano et al., 2010). It is known to bind haem with the highest affinity during haemolysis with systemic haem overload (Tolosano et al., 2010, Fiorito et al., 2013, Dong et al.,

2013).

The human HPx gene is 12kb in size with 10 exons and 9 introns and is located on chromosome 11p15.4-p15.5. The protein, HPx is a 60kDa glycoprotein with about 20% of carbohydrate. HPx consists of two homologous domains of about 200 residues each, joined by 20-residue non-covalent link. The two domains combine to bind a haem molecule. On separation of the two domains, the N-terminal domain retains its ability to bind haem like intact HPx while the C-terminal domain could not but retains its ability to bind to HPx receptor. Both the N-terminal and C-terminal domains contain a beta-propeller and show about 25% homology in their amino acid sequence.

HPx is a fully sialylated glycoprotein with 4-5 bi-antennary carbohydrate chains depending on the species. Human HPx is differentiated by its O-glycosylated threonine at the N-terminus. The carbohydrate moiety is apparently not involved in haem binding (Delanghe and Langlois, 2001). The only known receptor for the haem-HPx complex is the scavenger receptor, the Low Density Lipoprotein Receptor-related Protein-1 (LRP) also known as CD91. This receptor is expressed in many cell types, indicating multiple sites of haem-HPx complex recovery. The major sources of haem are haemoglobin (Hb) from ruptured erythrocytes, myoglobin and enzymes with haem prosthetic groups including peroxidases and cytochromes from damaged tissues or secreted myeloperoxidase from neutrophils.

HPx binding of Hb-derived haem is especially important when Hp is depleted or absent (Delanghe and Langlois, 2001, Schaer et al., 2014). The normal host response to intravascular haemolysis is an important scavenger-receptor-enzyme system (Hp-CD163-HO-1) that prevents the fHb/free-haem-induced toxicity. It is a homeostatic mechanism that increases the expression of Hp, CD163 receptors and Haem-oxygenase 1; HO-1 (Mendonca et al., 2012).

However, the plasma concentration of Hp is a limiting factor in some circumstances (Thomsen et al., 2013).

The depleted level of Hp could induce the accumulation of free-haem. This could occur when fHb is oxidized by Reactive Oxygen and Nitrogen Species (ROS and RNS) to methaemoglobin or by spontaneous breakdown of fHb (Ferreira et al., 2008). Free-haem is highly cytotoxic particularly to endothelial cells (Ferreira et al., 2008, Quaye, 2008). The toxicity induced by free-haem is believed to activate the second scavenger-receptor-enzyme system; HPx-CD91-HO-1, whereby the HPx scavenges free-haem to the hepatocyte through the CD91 receptor for further degradation by the haem-oxygenase-1 enzyme; HO-1 (Ferreira et al., 2008, Thomsen et al., 2013). The binding of haem to HPx in the plasma is in the ratio of 1:1 (Shipulina et al., 2000). About 90% of the HPx-haem complex is internalized in the parenchyma cells of the liver by receptor-mediated endocytosis within 2 hours (Immenschuh et al., 1995). This uptake of haem-HPx further stimulates intracellular protective mechanisms, including the induction of HO-1 (Paoli et al., 1999), which further degrade the haem in the cytoplasm of the liver. Free-haem when bound to HPx could influence the pathological process through gene expression, cellular signalling and nervous/immune-modulation (Fiorito et al., 2013).

HPx is widely involved in the modulation of a variety of physiological and pathological processes; studies indicated that HPx plays a role as an anti-oxidant, anti-apoptosis, immune regulation, and organic protection, and participates in the regulation of cell differentiation and extracellular matrix reconstruction (Dong et al., 2013). HPx provides neuroprotection in mouse models of stroke and intra-cerebral haemorrhage. It protects neurons in vitro against haem or ROS toxicity via haem oxygenase-1 (HO1) activity (Hahl et al., 2013, Vinchi et al., 2013).

HPx has been shown to have an anti-inflammatory function by suppressing

macrophages from releasing excess proinflammatory cytokines (Lin et al., 2012, Hahl et al., 2013). A correlation was also found between HPx level and the potential of sera to block the invasion of the hepatocytes by *Plasmodium falciparum* sporozoites (Pied et al., 1995, Delanghe and Langlois, 2001).

Despite the lack of an international reference range as a standard for HPx levels (Delanghe and Langlois, 2001), its circulatory plasma concentration is approximately 0.4 to 1.5g/l but fluctuates in certain pathological conditions such as haemolytic anaemia (Muller-Eberhard et al., 1968, Kamboh and Ferrell, 1987). The normal level in children was reported to be about 20% lower than that of adults (Kanakoudi et al., 1995). About 2mg/l of HPx is reported to be present in the urine of normal individuals (Lizana and Blad, 1983). The level detected in cerebrospinal fluid (CSF) is 1.8-3.4mg/l (Schuller et al., 1971).

HPx depletion is not as pronounced as that of Hp. Low levels of HPx therefore signify a severe or chronic degree of haemolysis (Langlois and Delanghe, 1996). Determination of HPx in tandem with Hp has potential applications in the laboratory assessment of intravascular haemolysis, particularly in populations characterized by lower reference values of plasma Hp (Delanghe et al., 1998b, Kasvosve et al., 2000, Delanghe and Langlois, 2001) because HP x allows monitoring of severe haemolysis when Hp is depleted. This is because HPx is recycled; released back into the circulation unlike Hp, which is not recycled (Delanghe and Langlois, 2001).

Hp usually has a higher level of acute phase responsiveness compared to HPx (Delanghe and Langlois, 2001).

1.6.6.3 Haem oxygenase-1

Haem oxygenase-1 (HO-1) is an inducible enzyme that degrades haem to carbon monoxide and biliverdin (Schluesener et al., 2001). It is an important

enzyme in the host response to intravascular haemolysis and also prevents the deleterious effect of haem (Mendonca et al., 2012), (Fig. 1.4).

HO-1 is induced by the release of haem (Schluesener et al., 2001) complexed with its scavenger receptor proteins as earlier mentioned. Prostaglandin D2 is also known to induce the expression of HO-1 (Kuesap and Na-Bangchang, 2010) as well as NO. NO protects against CM by inducing the production of HO-1 (Jeney et al., 2014).

Mice deficient in HO-1 had complete hepatic failure. HO-1 is cytoprotective in that it suppresses the pro-oxidant effect of free haem, which sensitizes the hepatocyte to undergo TNF-α mediated self-death (Seixas et al., 2009, Gozzelino and Soares, 2014); protects against inflammation; oxidative stress and restores liver function during and after parasite clearance (Dey et al., 2014). High levels of HO-1 expression reduce hyperglycemia, improve glucose metabolism, and partly protect renal tissue from hyperglycemic injury, possibly through its anti-oxidant role (Ptilovanciv et al., 2013).

The role of CD163, HO-1 and Hp in haem metabolism needs further investigation. The validation of their roles in malaria within a larger cohort might provide useful resources for the development of future targeted therapies that will be of importance in alleviating the malaria burden (Hansson et al., 2015).

1.6.6.4 C-reactive protein (CRP)

CRP is a major acute phase protein (Ansar et al., 2006) whose concentration increases significantly in infectious disease (Pied et al., 1989). It is secreted by the hepatocyte following stimulation by IL-1 (Nussler et al., 1991).

CRP is often present in general infection and trauma (Gillespie et al., 1991, Upragarin et al., 2005) but its presence in malaria in the absence of other ailments is indicative of the pathological process. A positive correlation has been reported

between plasma Hp, CRP and parasitaemia (Saad et al., 2013).

The plasma level of CRP is a surrogate marker of the degree of parasitaemia and malaria pathology (Taylor-Robinson, 2000). This is because its level correlates positively with parasitaemia unlike that of Hp (Hurt et al., 1994a).

CRP inhibits the development of sporozoites at an early stage of the infection (Pied et al., 1989). It prevents the parasite's hepatic development by preventing its movement in hepatocytes and by blocking parasite division through an antibody mediated process (Nussler et al., 1991). CRP is known to have a direct binding capacity for infected cells mediated by calcium and phosphorylcholine in pathological conditions.

In disease, CRP binding to infected erythrocytes is 2-3 fold stronger than to normal erythrocytes (Ansar et al., 2006). The effector function of the strong CRP binding to infected erythrocytes is demonstrated by its potency in activating the complement pathway (Ansar et al., 2006). The binding of CRP to erythrocytes also prevents the activities of some complement regulatory proteins such as CR1, CD35, CD55 and CD59, and enhances haemolysis that accounts for the pathogenesis of anaemia (Ansar et al., 2006).

There is lower level of CRP in children recovering from SMA than CM in areas of high transmission and this has been implicated as a risk factor for SMA in lower aged children (Waitumbi et al., 2004). On the other hand, the higher level of CRP in malaria was reported to be associated with death, hence the protein is a marker for malaria associated mortality (Paul et al., 2012).

Both CRP and parasitaemia are metrics for measuring the effects of intervention programmes like the deployment of insecticide treated nets (Hurt et al., 1994b). People using insecticide treated nets have lower levels of CRP and parasitaemia than people not utilizing insecticide treated nets (McGuire et al., 1996).

It appears that the development of partial immunity to malaria reduces the expression of CRP. Higher CRP levels were associated with parasitaemia in children under 1 year of age while lower levels were associated with parasitaemia in older children (Hurt et al., 1994a).

Recent studies showed that CRP levels negatively correlate with amounts of Zn and positively correlate with amounts of Cu in Sudanese patients with severe falciparum malaria (Saad et al., 2013), bringing to light the likely pathological role of mineral elements in malaria.

A (-286) C>T>A SNP in the promoter region of the CRP gene is known to influence CRP level and susceptibility to malaria. A significant number of malaria episodes and high parasite density are associated with the A allele but not the C and T allele (Giha et al., 2010).

1.7 Oxidative stress in malaria

The role of oxidative stress during malaria infection is still unclear. Oxidative stress plays a crucial role in the development of systemic complications in malaria. Malaria infection induces the generation of the hydroxyl radical ($OH^{\bullet}$), which most probably is the main reason for the induction of oxidative stress and apoptosis. Twice as much hydroxyl radical is produced by *falciparum* infected erythrocytes compared with normal erythrocytes (Atamna and Ginsburg, 1993).

A potential source of free radical production is the Hb molecule, since the parasite uses it as a source of amino acids for its own nutrition during the erythrocytic stage of the disease. This results in free haem release, which induces further intravascular oxidative stress, changes in erythrocytes, endothelial cells and parasite sequestration in tissues and organs such as the liver and the brain (Kumar and Bandyopadhyay, 2005).

Large amounts of ROS and RNS are products of macrophages and

neutrophils that are elicited in response to the infection. However, their continuous release offsets the balance between the oxidants and the antioxidants (Cabrales et al., 2011, Percario et al., 2012). During a malaria episode, a high level of oxygen radical is being produced as superoxide anions, which can kill the parasite but also cause tissue damage. To prevent this damage the antioxidant enzyme, Zn/Cu superoxide dismutase (SOD-1) converts these radicals into hydrogen peroxide, which can then be removed by catalase and the glutathione system. SOD-1 level has been related to tissue damage and is also a marker for oxidative stress (Andrade et al., 2010).

Malarial infection may be associated with oxidative damage and reduced alpha-tocopherol reserves in the erythrocyte membrane, suggesting that local antioxidant depletion may contribute to erythrocyte loss in severe malaria (Griffiths et al., 2001). Increased production of ROS by the parasite may deplete the erythrocyte of its defense mechanisms namely, SOD, catalase, glutathione peroxidase, NADPH, NADH, glutathione (GSH) and glutathione reductase (Mishra et al., 1994).

1.8 Cytokines in malaria

So far, the role of cytokines has been mentioned in several contexts. It is not possible to discuss infection without mentioning the role of cytokines that participate in cellular signaling. Cytokines are released from White Blood Cells (WBC), particularly T-lymphocytes expressing CD4 on their surface called T-helper cells (Th-cells) and classified as Th-1 and Th-2 (Berger, 2000).

Th-1 cells tend to produce pro-inflammatory cytokines such as IL-1, IL-6, IL-2, IL-12, IL-8, IL-18, TNF-α, and IFN-Y. Excessive production of these pro-inflammatory cytokines may lead to tissue damage or disease progression and needs to be countered by the anti-inflammatory cytokines of Th-2 cells such as IL-

4, IL-10, IL-5, IL-9, IL-1ra, TNF-α binding protein, sIL-1R and IL-13 (Berger, 2000, Goncalves et al., 2010). Other molecules within the above groups such as IL-2, IL-3, IL-7, IL-10, IL-11, IL-12 and Granuloma-Monocyte Colony stimulating Factor (GM-CSF) can also act as positive or negative growth factor in a number of cells (Gruys et al., 2005).

An early pro-inflammatory cytokine response helps in limiting the infection by inhibiting the growth of malarial parasites at low concentrations. On the other hand, if these pro-inflammatory responses are not down-regulated they result in disease progression and complications (Clark et al., 2006). IL-10 is an anti-inflammatory cytokine produced mostly by monocytes and T-cells in response to increasing levels of pro-inflammatory cytokines. It is responsible for the switching from a Th-1 to a Th-2 cytokine response; the biologic function of IL-10 is to inhibit the activity of Th-1 cells, natural killer cells and macrophages to counter the effect of pro-inflammatory cytokines (Goncalves et al., 2010).

Th-1 cells and their cytokines are important in parasite clearance but induce severe tissue damage at the same time. The action of IL-10 on these cells and also on the production of chemokines (MCP-1, and 5, IL-8, IP-10 and MIP-2) may prevent them from enhancing the pathological process (Couper et al., 2008). The biological functions of IL-10 are similar in various infections irrespective of its source of production (Goncalves et al., 2010).

The balance between the pro- and anti-inflammatory cytokines is important in malaria (Mackintosh et al., 2004, Naik et al., 2000). The ratio of IL-10 and TNF-α or IL-6 has been a parameter for measuring the balance between pro- and anti-inflammatory cytokines. This balance is proposed to exacerbate or ameliorate pathology. The induction of acute phase protein by IL-6 should down-regulate the actions of pro-inflammatory cytokines such as TNF-α and IL-1 (Gadient and Patterson, 1999). As a result, IL-6 appears to act both as a pro- and an anti-

inflammatory cytokine. Circulatory level of TNF-α, IL-6, and IL-10 and the ratio of IL-6:IL-10 were higher in dead patients than in survivors. Survivors have stable levels of both IL-6 and IL-10 but in most cases the level of IL-6 is lower (Day et al., 1999). Raised IL-10 and IL-6 have been associated with hyperparasitaemia, shock, and jaundice (Day et al., 1999).

Low IL-10 has been reported to be associated with SMA (Day et al., 1999). A study in a Nigerian cohort reported a very high level of IL-10 in UM and a lower level in SMA groups (Burte et al., 2013). In contrast, a study in Mali showed that both IL-10 and IL-6 are significantly higher in SMA compared to UM and healthy individuals (Lyke et al., 2004). Due to the disparity in these reports, it was considered that something else may account for the low level of IL-10 in the SMA group. In this study, it was hypothesized that Hp phenotypes are accountable for the disparity. Therefore, the relationship between Hp phenotypes and plasma cytokines level were investigated further.

1.9 Aims and objectives

This study is to investigate changes in the levels of plasma proteins with convalescence in *P. falciparum* malaria infected child cohort, living in the malaria-holoendemic city of Ibadan, Nigerian.

The human proteome is central for disease diagnosis, prognosis and therapeutic monitoring. The blood plasma contains a huge number of proteins derived from organs and tissues. Hence, it's a reflection of both the physiological and pathological state of cells (Saffer et al., 2002) and a good reservoir of disease-specific biomarkers (Liu et al., 2014). A unique characteristic of the plasma proteome is that the proteins are present in unique amounts; they are in a dynamic range to one another; their levels in a pathological condition differ from one ailment to another and they are more useful for objective assessment of the

disease state than clinical observation (Gillespie et al., 1991). All of these features made the plasma a very good sample for clinical investigation (Yu et al., 2011).

This study was initially a discovery-based project with the overall aim of looking into the differences in the levels of plasma proteins as well as proteins that are specific to each malaria syndromes and which could possibly explain the development of severe forms of malaria. It is not clear why some children develop severe malaria complication while the infection is restricted to the mild or uncomplicated form in others.

Specifically, the study aims;

1. To investigate and validate changes in the plasma proteins of children recovering from SMA in order to further understand the pathophysiology of SMA and how it differs to other syndromes and to search for and/or validate proteins associated with clinical malaria syndromes that could be useful as biomarkers for malaria diagnosis or prognosis. Monitoring the changes in the plasma protein through convalescence in comparison with the control will both help to understand the pathology of the disease and to identify potential biomarkers. The problem is that malaria in the sub-Saharan Africa is still poorly diagnosed due to lack of trained physicians and scientists; symptom overlap with other diseases and the absence of biomarkers that can discriminate among the clinical malaria phenotypes. Some of the clinical observations used to establish the diagnosis of malaria are not specific. For example, cerebral malaria is often defined with the presence of asexual form of the parasite, convulsion and coma. However, coma and convulsion are associated with other encephalopathy-like syndromes such as meningitis. Also, the presence of the parasite in the blood is not uncommon in these subjects as malaria is holoendemic. Lack of definitive diagnosis could leads to a high rate of antimalarial drug abuse; drug

resistance and high mortality rate, particularly among children less than 5 years of age. Therefore, the search for proteins that could effectively discriminate among the malaria syndromes and between malaria and other causes of fever at acute onset is important.

2. Following preliminary investigations on pool samples, we found that the levels of Hp, clusterin (CLU) and HPx were differentially expressed in the malaria groups relative to the levels in the healthy community controls (CC) and need to be investigated further on individual crude samples. This further led to a hypothesis-based study to address some specific questions such as;

- ❖ Are low levels of Hp in malaria a risk factor or a consequence of the malaria infections?
- ❖ How do the levels and isoforms of Hp in malaria affect the clinical outcome of the disease?
- ❖ Do individuals with different Hp phenotypes but presenting with the same malaria syndrome elicit the same response to the disease?
- ❖ Are there any genetic variants that are associated to both the disease and Hp levels.
- ❖ Are there significant changes in the plasma CLU and HPx levels in malaria syndrome between acute onset and convalescence?

CHAPTER TWO

2.0 MATERIAL AND METHODS

2.1 Total Protein Estimation Assay

Protein estimation assay was performed using bicinchoninic acid (BCA) and the manufacturer's (Pierce™) manual. Briefly, the BSA standard and the samples were diluted in lysis buffer (7.5mM Tris, 2M urea and 1% CHAPS), samples were diluted 1 in 200 in the buffer. Working reagent was prepared by mixing 50 part of BCA reagent A with 1 part of BCA reagent B. Standard and samples (25µL) were added to the plate followed by 200µL of working reagents. Reagents and samples were mixed on a plate shaker for 30 seconds and plates were incubated at 37°C for 30 minutes. Thereafter, plates were left at room temperature for 10 seconds and the absorbance was read at 560nm in a FLUOstar Omega plate reader (BMG-Labtech). Blank corrected values were plotted against concentrations in a standard curve and the unknown total protein concentrations were determined from the standard curve (linear regression fit) and multiplied by the dilution factor.

2.2 Two-Dimensional-Difference-Gel Electrophoresis (DIGE)

For the preliminary investigation, we performed plasma proteome profiling both crude and immunodepleted plasma samples. Briefly, thirty plasma samples collected from children between 6 and 156 months of age were pooled based on the clinical groups (disease versus control). The Seppro IgY14 spin column immunodepletion method was to remove the most plasma-abundant proteins including Albumin and Immunoglobulins (Igs). Typically, 120µg protein was resuspended at a final concentration of 10µg/µl in lysis DIGE buffer (30mM Tris, 8M urea, 4% CHAPS, pH8.5) and left on ice for 10 minute. A total of 960pmol CyDye (GE Healthcare reconstituted in DMF) was added and incubated 30 minutes at 4°C. The reaction was stopped within 10 minutes of adding 10mM

L-Lysine. Cy5 and Cy3 were used to label two different disease groups, whilst Cy2 was always used to label a pool of all disease groups (namely CC, UM, SMA and CM) to allow comparison between gels. For each gel, all three labeled samples were mixed together and buffered in DIGE isoelectrofocusing (IEF) buffer (8M urea, 4% [w/v] CHAPS, 0.4% [v/v] carrier ampholyte, 0.0004% [w/v] bromophenol blue, 130 mM DTT) to a total of 200µl. Samples were applied onto ReadyStrip IPG strips (pH 3-10, 11 cm, Bio-Rad, UK) and actively rehydrated for 13 hours 40 minutes at 50 V followed by the IEF (250 V for 15 min linear, 8,000 V for 2 hours linear, 8,000 V for 35,000 V/hour rapid) using a PROTEAN IEF cell (Bio-Rad, UK).

After IEF, the IPG strips were immediately equilibrated: strips were transferred to 2% (w/v) DTT in equilibration buffer (6M urea, 2% (w/v) SDS, 20% (v/v) glycerol, 50 mM Tris pH 8.8) for 15 minutes followed by another 15 minutes in 2.5 % (w/v) iodoacetamide in equilibration buffer. Thereafter, strips were then processed to SDS-PAGE.

2.2.1 SDS-PAGE: Second Dimension Electrophoresis

Bio-Rad precast Criterion gels that accommodates 11cm strips and running buffer (1x Tris/Glycine/SDS) were used in the second dimensional gel. The IPG strip was held gel side up and the plastic backing was placed against the back of the gel cassette. The strip was centered at the middle of the gel and pressed slightly with the aid of the forceps until it firmly touched the gel.

Paper dipped in Precision Plus Protein standard ranging from 10kDa to 250kDa was placed into the designated well on the right side of the gel with the aid of forceps. Overlay agarose (Bio-Rad) was used to seal the precision plus standard and the strip. After the agarose has solidified, the electrophoresis was performed in the criterion cell, connected to power supply at 200V (constant) for an hour.

2.2.2.Two-Dimensional (2D) Gel electrophoresis (2DE)

120µg plasma protein containing a pooled sample of all disease groups was resuspended in 125µL of sample IEF rehydration buffer (8M urea, 4% [w/v] CHAPS, 2% [v/v] carrier ampholyte, 0.0002% [w/v] bromophenol blue, 65 mM DTT) and shaken for 2 h at room temperature. Samples were applied onto ReadyStrip, 11 CM IPG strips (Bio-Rad, U.K.) and actively rehydrated for 13 h and 40 min at 50 V. Thereafter, the IEF (250 V for 15 min linear, 4000 V for 2 h linear, 4000 V for 10 000 V/h rapid) was performed in a PROTEAN IEF cell (Bio-Rad, U.K.).

After the IEF, strips were either stored at −80 °C or processed immediately. For equilibration, strips were transferred to 2% (w/v) DTT in equilibration buffer followed by 15 min in 2.5% (w/v) iodoacetamide in equilibration buffer. The proteins were further fractionated by SDS-PAGE (as described above). The gel was rinsed trice in water for 10 minutes to remove the SDS and then stained for an hour in Coomassie, CooBlue stain (Interchim) The Coomassie stained gel was then destained in water until a clear background was achieved.

2.2.3 Gel Analysis

The gels were analyzed with Bio-Rad PDQuest. Differentially expressed spots were selected if showing a change of more than 1.5-fold expression between two groups; disease versus control as quantified by the PDQuest (Bio-Rad). The Coomassie stained gel was aligned to DIGE images to select spots for excision. The PDQuest (Bio-rad) software was also used to excise spots of interest using EXQuest spot cutter.

2.2.4 Mass Spectrometry Analysis

Excised spots were washed in 200mM ammonium bicarbonate in 50% acetonitrile (ACN) for 30 minutes at 37°C. This step was repeated 3-5 times until spots were completely de-stained. The spots were incubated in 20mM DTT (prepared in 200mM ammonium bicarbonate in 50% ACN) for an hour at 37°C followed by a wash without DTT. Spots were then incubated in 5mM iodoacetamide (prepared in 200mM ammonium bicarbonate in 50% ACN) for 20 minutes at 37°C. Spots were further washed twice in 20mM ammonium bicarbonate in 50% ACN and supernatant removed. ACN (100%) was added for 15 minutes until spots were shrunk opaque, the supernatant was again removed and spots were left to dry.

In-gel digestion was performed by adding 2ug/ml Trypsin Gold (Promega) and then left at 32°C for 16 hours to recover the spot. Trypsin supernatant was then processed to LC-MS/MS analysis using the OrbiTrap LC-MS (ThermoScientific). Peptide profile was extracted and transferred to the Mascot database for protein identification.

2.3 Immuno-probing Assays for Hp

Western blotting and immuno-probing were performed in order to determine the different Hp phenotypes in the population. After the initial separation of the proteins using the SDS-PAGE, the proteins were transferred to a nitrocellulose or PVDF membrane by western blotting before the immuno-probing assay.

2.3.1 SDS-PAGE

The volume of plasma sample corresponding to 20µg of total protein or 10,000ng/mL of Hp as determined from ELISA was diluted in lysis buffer (7.5mM Tris, 2M urea and 1% CHAPS) and further diluted 1:1 in Laemmli buffer (Bio-Rad)

containing the recommended volume of β-mercaptoethanol (BME). The proteins were then denatured in the heat block at 98°C for 5-10 minutes. Thereafter, tubes were briefly centrifuged before loading the protein into the gel. A 16% gel made from both resolving gel [30% acrylamide stock, resolving buffer (1.5M Tris buffer, pH 8.8 and 10% (w/v) SDS), distilled water, 10% or 50µl of ammonium persulphate; APS and 10µL TEMED] and stacking gel [30% acrylamide, stacking buffer (0.5M Tris buffer, pH6.8, 10% SDS w/v), distilled water, 50µL APS and 10µL TEMED] was used. The electrophoresis was performed in 1X Tris/glycine/SDS buffer (geneflow^{R}) at 120V for the first 20 minutes and 150V for the second 25 minutes.

2.3.2 Western Blotting

Blotting to PVDF or nitrocellulose membrane was performed in a Bio-Rad trans-blot cell using electro-blotting buffer (39mM glycine, 48mM Tris-base, 0.0375% SDS, 20% methanol and distilled water). The sandwich was prepared in a gel holder cassette. Briefly, a pieces of fiber presoaked in the electro blotting buffer is placed on the clear panel of the cassette followed by pre-soaked pieces of filter paper that is about the size of the gel. A PDVF or nitrocellulose membrane was soaked briefly in methanol and in the electro-blotting buffer and was placed on top of the pre-soaked filter paper, taking care to avoid air bubbles. Thereafter, the gel was placed on the nitrocellulose or PVDF membrane. The sandwich was completed by placing a piece of filter paper on top of the gel followed by another pre-soaked fiber pad. The sandwich cassette was then closed and placed in the buffer tank with the clear panel facing the red anode. Electro-blotting buffer was added up to the recommended mark on the tank. The cell was connected to the power supply at a constant voltage of 30V overnight (approximately 15-16 hours). Thereafter, the nitrocellulose was removed and stained with ponceau S prior to the

blocking step in order to briefly visualize the transferred protein.

2.3.3 Immuno-probing for Hp

The membrane was placed in 10mL blocking solution [2-3% marvel milk in PBS-tween (1X PBS and 0.1% Tween 20)] for an hour. Thereafter the membrane was incubated in polyclonal rabbit anti-human Hp (Dako-Cytomation) primary antibody (diluted 1 in 10,000 in blocking solution) with mild shaking for 1 hour at room temperature or 4^0C overnight. Thereafter, the nitrocellulose was washed thrice in PBS-tween for a minimum duration of 20 minutes at room temperature and was incubated at room temperature for 2 hours in fluorescent goat anti-rabbit IgG secondary antibody (Alexa Flour^R 488) diluted 1 in 5000 in PBS-tween. Another washing step was performed and the nitrocellulose was rinsed in water. The Pharos FX^{TM} Plus Molecular Imager (Bio-Rad) was used to scan the blot for the protein (Hp) bands.

Alternatively, goat anti-rabbit IgG HRP-conjugated secondary antibody solution was used. The membrane was incubated for an hour at room temperature with gentle shaking in HRP-conjugated secondary antibody diluted 1 in 10,000 in blocking solution. Thereafter, the blot was rinsed in fresh wash buffer for 5-10 minutes with gentle agitation.

For chemi-luminescent detection; an equal volume of HRP-substrate solution, 3mL luminol reagent and 3mL peroxide solution mixed in a clean container and allowed to stand for 10 minutes in the dark was used to incubate the membrane for 5 minutes at room temperature. Thereafter, the blot was removed and was covered in a clean plastic wrap avoiding air bubbles. It was then exposed to X-ray film in the dark for 10-30 seconds and the film was developed for band visualization.

2.4 Enzyme-Linked Immunosorbent Assays (ELISA)

ELISA was used for the accurate quantification of some plasma proteins of interest. All the standard curves for the assays described below are found in the Appendix 1.

2.4.1 Quantitative Determination of Plasma Hp

Hp level in the plasma samples were quantified using an Human Hp Sandwich Enzyme-Linked Immunosorbent Assay (sandwich ELISA; sELISA) kit (Genway) and manufacturer's procedure with slight modification. Plasma samples were diluted 1/20, 1/500, 1/1000, 1/10,000 and 1/20,000 in phosphate buffered saline (PBS) to ensure the detection of any Hp present either at low or high concentrations in the plasma. Affinity purified Chicken IgY against Human Hp was diluted in coating buffer (0.05M sodium carbonate-bicarbonate, pH 9.6) to working concentration of 5µg/mL and was used to coat the plate by adding 100µL to each well of 96 well plates.

The plates were then incubated at room temperature for 1 hour or overnight at 4°C. Thereafter, 400µL of blocking solution (50 mM Tris-HCl, 140 mM NaCl, 1% BSA, pH 8.0) was added to each well and incubated for an hour at room temperature. Affinity purified human Hp used, as the standard was diluted 1 in 10 in PBS to a concentration of 200ng/mL as the stock. Serial dilutions at 1:3 were then made from the stock to give the lowest standard concentration of 0.27ng/mL.

One hundred microlitre (100µL) of both standard and samples were added to the respective wells excluding blank. The plates were incubated at room temperature for an hour. Thereafter, wells were washed five times with washing buffer (0.05% Tween 20 in PBS, pH 7.4). For each washing step, the wells were filled with washing buffer, swirled on the table and allowed to stand for a minute. Thereafter, the detection antibody (100µL), HRP conjugated chicken IgY anti-

human Hp diluted 1:2000 in conjugate diluent (50mM Tris, 0.14M NaCl, 1%BSA, 0.05% Tween 20, pH 8.0) were added to each well and incubated at room temperature for an hour. Another washing step was performed as described above. Thereafter, OPD substrate was prepared at 1 mg/mL by dissolving one OPD tablet (Acros Organics) in 20mL citrate-phosphate buffer (50mM citrate-phosphate with 30% of H_2O_2 in distilled water) and 100µL of it was added to the respective wells and the plate was incubated for 10-30 minutes.

A stop solution, 2M H_2SO_4 (100µL) was added to stop the OPD reaction after a considerable colour formation within 15-20 minutes. Plates were read for absorbance at 490nm with a FLUorStar-Omega ELISA plate reader (BMG-Labtech). A MasterPlex ReaderFit v2.0 software (MiraiBio Group, Hitachi Solutions America, Ltd.) was used to generate a five to four parameter logistic (5/4-PL) standard curve-fit and the concentrations of the unknown were determined from the standard curve.

2.4.2 Quantitative measurements of plasma Hp1-1, Hp2-1 and Hp2-2

All the samples were separated based on their Hp genotypes or phenotypes. Sandwich ELISA was performed to quantify the level of each phenotype of the protein. Human Hp containing the appropriate amount of its entire sub-units (α1, α2-chains and the β-chain) was used as the standard. All other procedures were the same as those of Hp sELISA described above. The phenotypes were randomly quantified together in order not to introduce variation.

2.4.3 Quantitative measurement of plasma Hemopexin (HPx) levels

Plasma levels of HPx were measured using the human HPx sELISA kit (Genway) and the manufacturer's instruction. The anti-human HPx antibody was adsorbed to the surface of polystyrene microtitre wells. Standards or calibrators

diluted 1 in 2 from 200ng/mL to 6.25ng/mL and samples diluted 1 in 40,000 were added to the designated wells. After the washing steps, anti-HPx-HRP conjugate was added for 2 hours and was followed by another washing steps. The enzyme bound to the immunosorbent was assayed by adding 3,3,5,5, tetramethylbenzidine (TMB). The amount of bound enzyme varies directly with the concentration of HPx in the samples. The absorbance was read at 450nm. The standard curve and the concentration of unknown were determined as mentioned in section 2.4.1.

2.4.4 Measurement of human plasma free Haemoglobin (fHb) levels

Plasma fHb was assayed using Human-fHb ELISA kit (MyBioscience) and manufacturer instruction. The standards were diluted 1 in 2 from 1000ng/mL as the highest concentration to 15.625ng/mL as the lowest concentration. Samples were diluted 1 in 800 in the same diluent. The assay employed direct competitive inhibition enzyme immunoassay technique. Briefly, antibody specific to haemoglobin (Hb) was coated to the plate. 50µL of standards, samples and biotin-conjugated Hp were pipetted into the wells and incubated at 37°C for 40 minutes. A competitive inhibition reaction was then launched between Hb (standard or samples) and biotin conjugated Hb for the precoated Hb antibody. After the washing steps, 100µL of avidin conjugated HRP was added and incubated at 37°C for 40 minutes and was followed by another washing step. A substrate solution, 90µL of TMB was added and incubated at 37°C for 20 minutes. Thereafter, 50µL of stop solution was added and the absorbance was read at 450nm with wavelength correction at 540nm. The colour formed was inversely proportional to the amount of fHb bound in the initial step. Unknown concentrations were determined from the standard curve as earlier mentioned in section 2.4.1.

This assay was designed to avoid the measurement of free haem in the plasma, as free haem does not have the globin moiety that is the target of the

monoclonal antibody.

2.4.5 Quantitative measurement of Plasma Lactate Dehydrogenase (LDH)

The level of LDH was measured by sELISA using human LDH ELISA kit (Cloud-clone Corp) and the manufacturer instructions but with slight modifications. Plasma samples were diluted 1:200 in PBS while the standard was diluted 1 in 2 ranging from 10ng/mL to 0.156ng/mL. Plates were read at 450nm and the concentrations were calculated from the standard curve as earlier described in section 2.4.1.

2.4.6 Quantitative measurement of sCD163 by ELISA

Mouse anti-human CD163 (360µg/mL) reconstituted in PBS to 2µg/mL was used as the capture antibody and the biotinylated mouse anti-human antibody (180µg/mL) reconstituted in reagent diluent (1%BSA in PBS) to 1µg/mL was used as the detection antibody while the human CD163 (520ng/mL) was used as the standard. Both the antibodies and the standard were purchased from R&D. The standard was diluted 2-fold in reagent diluent with concentration ranging from 20,000pg/mL to 312.5pg/mL and the samples were diluted 1 in 100 in the same diluent. Tween 20 (0.05%) in PBS, pH7.2 was used as the wash buffer, TMB reagents (R&D) and H_2O_2 mixed 1:1 was used as substrate solution and 2N H_2SO_4 was used as the stop solution. The ELISA was performed following the manufacturer instruction. The concentration of the unknowns was determined from the standard curve as described in section 2.4.1.

2.4.7 Quantitative measurement of Plasma Clusterin (CLU)

The human plasma CLU levels were quantified using human CLU ELISA kit (Ray Biotech. Inc.) and the manufacturer's manual. The plasma samples were

diluted 1 in 50,000 in 1X assay diluent. A 2-fold dilution was performed for the standard with concentrations ranging from 20,000pg/mL to 19.53pg/mL. Both standard and samples (100µL) were added to the designated wells of the 96 wells plate that have been coated with human anti-CLU antibodies. The plate was then incubated at 4°C overnight with gentle shaking. Unbound analytes were removed in the washing steps. Biotinylated antibody (1x) was added to the plate and incubated for 1 hour at room temperature. After another washing steps, the HRP-streptavidin solution was added for 45 minutes followed by another subsequent washing steps to remove the unbound anti-CLU-HRP. The amount of CLU bound to the enzyme-labelled antibody was assayed by adding TMB. After 15-20 minutes, the reaction was stopped with 50ul of the stop solution. The concentration of the unknowns was determined from the standard curve as described in section 2.4.1 above.

2.4.8 Quantitative measurement of Plasma C-reactive protein (CRP)

The plasma level for CRP was quantified using CRP human ELISA kit (Invitrogen) and the manufacturer's instruction to measure the acute phase responsiveness. The plasma was diluted 1 in 50,000 and the standard was diluted in 2-fold with concentrations ranging from 1200pg/mL as the highest standard to 18.75pg/mL as the lowest. The concentration of unknown was determined from the blank corrected absorbance and the standard curve as described in section

2.4.9.Quantitative measurement of plasma SOD-1 levels by ELISA

The plasma levels of soluble superoxide dismutase-1 (SOD-1) were measured by the method of ELISA using a commercialized human SOD-1 ELISA kit (Cloud-clone Corp) and the manufacturer instructions. Polyclonal antibody against human SOD-1 was used as the primary antibody. The plasma samples

were diluted 1 in 500 in PBS. A stock of 4,000pg/mL was made from the standard (32,000pg/mL as supplied) and was further diluted 1 in 2 in PBS to the lowest standard concentration of 62.5pg/mL. The absorbance measurement and determination of unknown concentrations were as described in section 2.4.1.

2.5 Human Free-haem Assay

The free-haem was measured by colorimetric assay using haemin colorimetric assay kit purchased from BioVision Inc. and the manufacturer's manual. The kit utilizes peroxidase activity in the presence of free-haem, even in trace amount, to produce colour change measurable at 570nm. Briefly, the plasma samples were diluted 1:1000 in the haemin assay buffer. Hemin standard was also diluted in haemin assay buffer to 40, 80, 120, 160, and 200nM. Both sample and standard were incubated with the kit reaction mix (prepared from enzyme mix, haem substrate, Assay buffer and probe in the DMSO as supplied) for 30-60 minutes. The amount of free haem in the plasma was quantified from the blank corrected absorbance and the standard curve.

2.6 Cytokine Multiplex Assay

Human plasma cytokines were measured using the human cytokine 17Bio-plex beads immunoassay kit (Bio-Rad) and the manufacturer's instructions. Samples were diluted 1 in 5 prior to mixing with magnetic beads. The machine was calibrated prior to the measurement and plates were read using the Bio-Plex 200 Systems (Bio-Rad). The amounts of analytes as assayed in each of the samples were analyzed using the Bio-Plex Manager 6.0 software (Bio-Rad Laboratories) or the MasterPlex ReaderFit v2.0 software (MiraiBio Group, Hitachi Solutions America, Ltd).

2.7 Hp Genotyping by PCR

Genotyping was performed particularly when there was null to low level of plasma Hp, for those subjects without plasma samples but only DNA samples and to confirm the phenotypes that was determined by western blotting in case of antibody cross-reactivity with Haptoglobin Related Protein (HPR).

A forward primer, Sa5 (5' GAGGGGAGCTTGCCTTTCCATTG 3') and reverse primer, Sa6 (5' GAGATTTTTGAGCCCTGGCTGGTG 3') were designed for both Hp1 and Hp2 alleles (Fig.2.1).

Other primers, such as the forward, LS1 (5' TGAGCACTTAAGAGAGCAGGC 3') and reverse, LS2 (5' CTTCACATTCAGGAAGTTTATCTCC 3') were designed for the specific genotyping of only the Hp2 allele (Fig.2.1).

The PCR mix used for LS1 and LS2 primers is as follows; Kapa HiFi HS DNA polymerase (0.5μL); 10mM Kappa DNTP mix (0.75μL); Kapa HiFi fidelity buffer (0.5μL); forward and reverse primers (10μM each); template DNA (50ng) and distilled water to a total reaction volume of 25μL.

The PCR was performed at initial denaturation, 95°C, 3 minutes and 35 cycles of second denaturation (98°C, 20 seconds), annealing (61.5°C, 15 seconds), and extension (72°C, 2 minutes). A final extension step was performed at 72°C for 2 minutes.

The PCR reaction for Sa5 and Sa6 is similar to that of LS1 and LS2 but with 100ng of template DNA, an additional 2mM of $MgCl_2$ and at an annealing temperature of 69.2°C or 71.5°C depending on the PCR machine (Eppendorf Mastercycler).

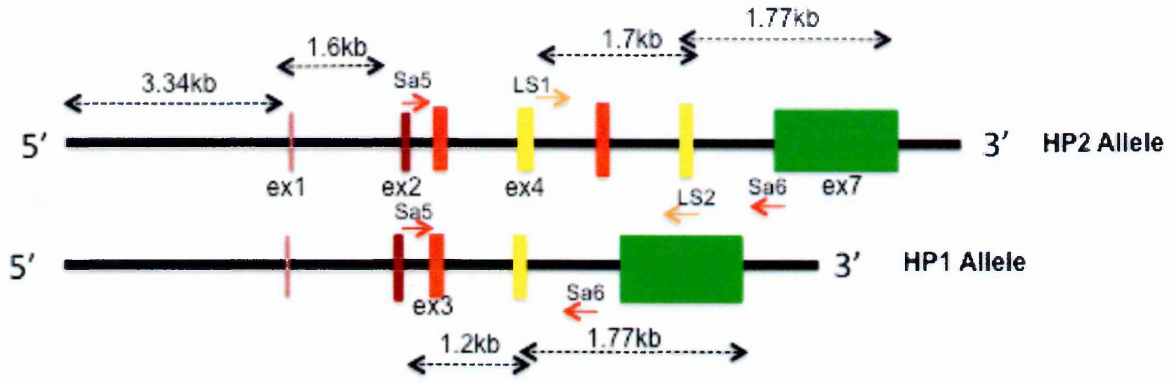


Figure 2.1 Primer mapping for Hp genotyping

The positions for the pairs of primers Sa5 and Sa6 are as indicated by the red arrows and the LS1 and LS2, which are specific for only Hp2 allele are indicated by the yellow arrows. Ex=exons, labeled 1 to 7. Both exon 3 and 4 are duplicated in the Hp2 allele. The Hp2 allele is 1.7kb longer than the Hp1 allele due to the duplication. The combinations of these primers were used for specific genotyping of Hp among subjects.

2.7.1 Gel Electrophoresis

To check for the right size of PCR products (amplicon), gel electrophoresis was performed with 1.0-0.7g of Agarose melted in 100mL 1X TAE running buffer. Ethidium Bromide (1µL) was added to the melted agarose and the running 1X TAE buffer. PCR product (12µL) mixed with loading dye (either Bioline or prepared) together with 1kb hyperladder (Bioline) was loaded into designated wells on the gel. The electrophoresis was performed mostly at 98V (higher voltage was dependent on the size of the tank, the distance between the electrodes of the tank, ~8-10V/cm) until the dye front reached the desired point depending on the size of the PCR product. The DNA was then visualized at 302nm using a UVP transilluminator.

2.8 High Throughput *Hp* gene sequencing

The *Hp* gene was sequenced using amplicon-based Nextera Illumina sequencing on a MiSeq machine. Overlapping amplicons covering the entire length of the Hp gene extending to 3.34kb upstream of the first exon were designed (Fig. 2.2) and generated by monoplex PCR. Both the promoter and the

distal upstream region of the promoter were covered in the design of the amplicons (Fig. 2.2).

In order to generate these amplicons per sample, monoplex PCRs was performed for each amplicon specific primer that was used (Table 2.1). This was because multiplex PCR failed due to differences in the Tm of the primers and the regions of the Hp gene; some regions were more difficult to amplify. A validated control was introduced into each set of PCR reaction to check for optimum performance of the PCRs.

2.8.1. DNA quantification assay

The concentration of DNA was quantified using the picoGreen fluorescence assay (Invitrogen), a more sensitive assay than the UV absorbance method. The assay utilizes a cyanide dye that fluoresces upon binding to dsDNA. Briefly, DNA standard was prepared in a 1x TE buffer (10mM Tris, 1mM EDTA, pH7.5) in a two-fold dilution to final DNA concentration of 2000ng/mL to 25ng/mL and containing 200-5ng of DNA per well. The DNA samples were diluted 1:100 in 1x TE buffer. Picogreen reagent (100µL) diluted 1:200 in the same buffer was mixed with both standard and samples and incubated 2-5 minutes at room temperature prior to measuring the fluorescence at 420nm excitation and 525nm emission wavelengths. The amount of DNA was quantified from the standard curve.

2.8.2 Preparation of Hp amplicon by monoplex PCR

Overlapping primers specific to different regions on the *Hp* gene were designed to produce overlapping fragments (amplicons) of the entire *Hp* gene (Fig. 2.2) in a series of monoplex PCRs. PCRs were performed for each of the 9 primers to generate 9 amplicons for each sample or patient. A total of 63 different PCR reactions were performed as earlier mentioned.

The amplicons from each pair of primers were pooled per well or samples after the right sizes of the amplicons were visualized by gel analysis; electrophoresis (as described in 2.7.1). The pooled amplicons were further used in the library preparation for Nextera Next Generation Illumina Sequencing on MiSeq.

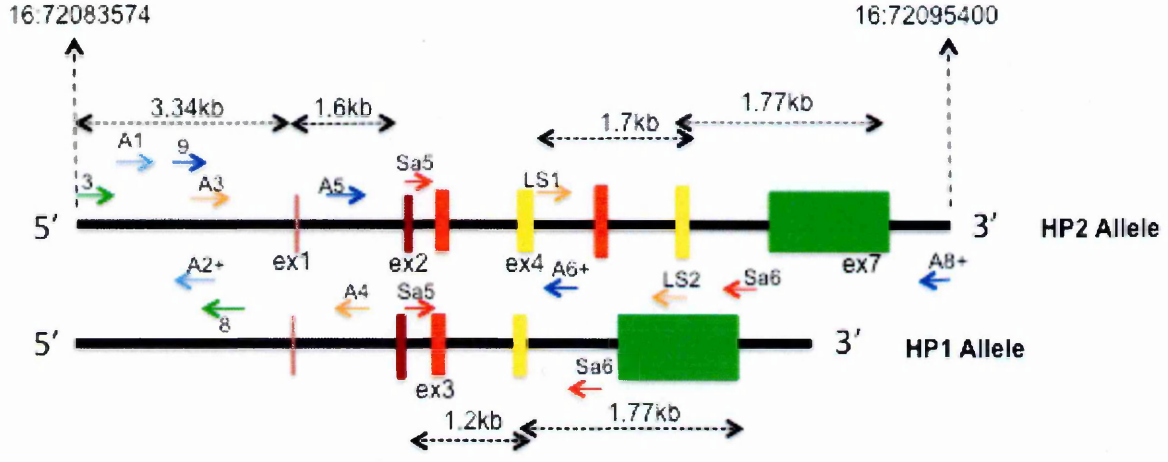


Figure 2.2 Primer mapping for amplicon amplification

Primers specific to the different regions of the Hp gene were designed to produce overlapping amplicons across the entire Hp length as indicated by dotted upward arrows, which indicate the start and stop position on chromosomes 16 covered by our primers. Short single arrows indicate the respective positions of primers. Ex1= Exon 1 etc, Single arrowheads indicate primers positions.

As mentioned earlier, the primers were designed to cover the *Hp* gene from 3.4kb upstream of the start codon to beyond exon 7 with the Hp2 allele and exon 5 with the Hp1 allele, and covering 16:72083574-72095400 in the GRCh37 or hg19 version of the human genome database (Fig. 2.2). The Hp2 allele from the GRCh37 version of the human genome database was used as the reference gene for the primer design. The primer recognizes corresponding regions of the Hp1 allele but shorter PCR products were produced for those primers covering the regions of difference/duplication between Hp1 and Hp2 alleles (Fig. 2.2).

The PCRs, particularly the annealing temperatures were determined after series of optimization for each primer in a gradient PCR. KAPA HiFi HotStart ReadyMix (2.5mM MgCl, 0.3mM of each dNTP, 1U of KAPA HiFi HotStart DNA polymerase per 50µL reaction volume) PCR kit (KK2502; KapaBiosystems) was

used in most cases except for LS1, LS2 and Sa5, Sa6 primers (KK2501; KapaBiosystems). Template DNA (50-100ng) was used for the PCR. The Tm_0 of both forward and reverse primers were designed to be as close as possible. There were no secondary structure formations by the primers. The %GC of the primers used ranges from 50-57.8.

All the designed primers were purchased from Sigma-Aldrich. On arrival, the lyophilized primers were reconstituted in distilled water (dH_2O) by adding the recommended volume to make 100µM. The primers were then further diluted 1:10 to a working concentration of 10µM. The final concentration of the primer in the PCR mix was 0.3µM in a 25µL reaction volume on adding 0.75µL of 10µM working concentration. Table 2.1 shows the list of primers, PCR mix and reaction.

2.8.4 Nextera^R XT DNA library preparation

The DNA quality and quantity was checked before commencing the process of library preparation. The minimum amount required for Hp sequencing on MiSeq is 1ng of DNA. The library was prepared as discussed below.

2.8.4.1 Amplicon quality and quantity check

Each amplicon (5µL) corresponding to each patient were pooled together following the working template. At the end, each well of the 96 well plates contained fully amplified overlapping fragments of Hp for each sample. The concentrations and quality of the pooled amplicons (DNA) were examined on a Glomax and Bioanalyzer prior to library preparation. All samples were of a good quality with concentrations between 22-100ng/mL on the average.

Table 2.1 List of primers, PCR mix and reactions

S/No	Primers	Sequence	Size (H2 allele)	PCR reaction	PCR mix
1	3 A4	5'TACTACCCAGCCCAAAAGCTGG 3'- Fwd 5' AGCATACCAAGCTTCCAGC 3'- Rev	4.371kb	1. 95°C, 3minutes 2. 98°C, 20seconds 3. For 3 and A4=59.7°C;	2x KAPAHiFi HotStart Ready Mix=12.5ul,
2.	A5 A8+	5' TTGGTATGCTCAGAAGCTGC 3'- Fwd 5' GAGCTAGCCTCTTCCTCAACC 3'- Rev	5.532kb	A5 and A8+ =62.8°C and 59.5°C; A1 and A6+ = 64.4°C	0.3µM FWD primer= 0.75µL 0.3µM Rev primer = 0.75µL
3	A1 A6+	5' CATCCTAGTAGGTGTGAAGTGG 3'- Fwd 5' AGGCTCTCTGTATGCACAGGC 3'- Rev	5.143kb	4. 72°C, 4minutes 5. 2 to 4=35 cycles 6. 72°C, 4.30minutes 7. Hold=10°C	Volume corresponding to 50-100ng of template DNA usually 0.5-5µL Made up to 25µL with dH_2O
4	A1 A4	5' CATCCTAGTAGGTGTGAAGTGG 3'- Fwd 5' AGCATACCAAGCTTCCAGC 3'- Rev	3.3346kb	1. 95°C, 3minutes 2. 98°C, 20seconds 3. For	This PCR mix applies to all the primers except LS1, LS2 and Sa5 Sa6
5	9 A6+	5' CAATAGGGAGATGGCCACACAC 3'- Fwd 5' AGGCTCTCTGTATGCACAGGC 3'- Rev	3.580kb	A1 and A4=59.7°C; 9 and A6+=66.1°C; A3 and A6+ = 61.5°C;	
6	A3 A6+	5' ACTCAAAATGAGCCTTTCTGC 3'- Fwd 5' AGGCTCTCTGTATGCACAGGC 3'- Rev	3.468kb	3 and 8 = 66.1°C; 4. 72°C, 3.30minutes 5. 2 to 4=35 cycles	
7	3 8	5'TACTACCCAGCCCAAAAGCTGG 3'- Fwd 5' CCCTGTTAAGAGTTGAGCTCTTGC 3'- Rev	2.910kb	6. 72°C, 4minutes 7. Hold=10°C	
8	LS1 LS2	5' TGAGCACTTAAGAGAGCAGGC 3'- Fwd 5' CTTCACATTCAGGAAGTTTATCTCC 3'- Rev	~1.7kb	Note: PCR reaction for LS1, LS2 and Sa5, Sa6 were as described in section 2.7	Note: PCR mix for LS1, LS2 and Sa5, Sa6 were as described in section 2.7
9	Sa5 Sa6	5' GAGGGGAGCTTGCCTTTCCATTG 3'- Fwd 5' GAGATTTTTGAGCCCTGGCTGGTG 3'- Rev	~3.4kb		

The table indicates the list of primers shown in Fig.2.2 and their respective amplicon sizes, PCR mix and the PCR reactions that was used.

2.8.4.2 Nextera XT DNA library preparation

For library preparation, a Nextera XT DNA sample preparation and index kits (Illumina) were purchased according to the total number of samples. The sample preparation kit include the Amplicon Tagment Mix (ATM), Tagment DNA buffer (TD), Nextera PCR Master mix (NPM), Resuspension buffer (RSB), Library Normalization Additive1 (LNA1), Library Normalization Wash1 (LNW1), Hybridization Buffer (HT1), Neutralize Tagment buffer (NT), Library Normalization Bead-1 (LNB1), Library Normalization Storage buffer 1 (LNS1), index primers S501-S508 and N701-N712. Prior to the onset of library preparation, the kits were stored as recommended. The recommended user supplied consumables were also supplied for the assay.

The Nextera sample preparation kits enable up to 24 or 96-library preparation with unique indexes to ensure a library with proper index combination in the multiplex pool. The Nextera XT DNA Sample Preparation Kit uses an engineered transposome to simultaneously fragment (enzymatic DNA fragmentation to ~300-500bp) and tag input DNA, adding unique adapter sequences in the process. A limited-cycle PCR uses these adapter sequences to amplify the insert DNA. The PCR also adds index sequences on both ends of the DNA, thus enabling dual-indexed sequencing of pooled libraries on Illumina MiSeq as diagrammatically represented below (Fig. 2.3).

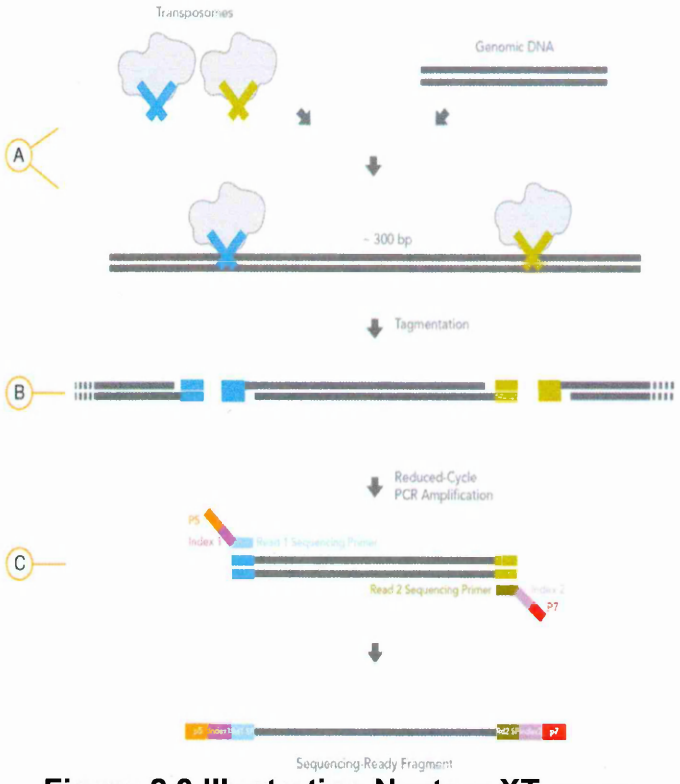


Figure 2.3 Illustrating Nextera XT assay

A. The illumina Nextera assay uses enzymes called transposomes to cut DNA into short segments and (B) at the same time adding adapters. (C) A reduced cycle PCR is used to add unique index. The overall process produces a sequencing ready fragment.

The libraries were prepared following the nextera XT DNA library preparation guide.

Briefly,

1. Tagmentation of DNA was performed with 5µl (1ng) input DNA, 10µl TD and 5µl ATM to allow amplification by PCR in subsequent step.
2. A limited PCR amplication step was performed with 15µl of NPM and 5µl of index primers S501-508 (i5) and N701-712 (i7) arranged on column A-H and row 1-12 respectively on illumina index plate fixture such that each sample had unique index sequences. The PCR was performed at 72°C for 3 minutes, 95°C for 30 seconds, and 12 cycles of: 95°C for 10 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and 72°C for 5 minute and then held at 10°C. This step ensured the amplification of tagmented DNA and addition of index i5 and i7 required for cluster formation.

3. A PCR clean-up step was performed with RSB, AMPure XP beads and 80% freshly prepared ethanol. This procedure was performed to purify the library and to remove very short library fragments from the population.
4. The LNA1, LNB1, LNW1, LNS1 and freshly prepared 0.1N NaOH and illumina procedure were used to normalize the library to ensure equal library representation in the pooled samples.
5. To generate sequence ready fragments, equal volumes of normalized libraries were combined, diluted in HT1 and heat denatured prior to MiSeq sequencing. Briefly, 5µL of each library to be sequenced were transferred column by column to a PCR 8-tube strip. Pipette tips were changed at each point to avoid sample cross-contamination. The content of the 8-PCR tubes were further combined into a single Eppendorf tube and labeled as Pooled Amplicon Libray (PAF), which was further diluted with 576µL of HT1 into another fresh Eppendorf tube labeled as Diluted Amplicon Library (DAL). A heat denaturation process was performed on the DAL at 96^0C for 2minutes and was mixed and kept on an ice-water bath for 5 minutes. Thereafter, the DAL was loaded into the MiSeq reagent cartridge in order to ensure efficient loading of the MiSeq flowcell for sequencing.
6. A trained technician sequenced the library in accordance with the MiSeq system user guide. A phred-scale quality score >30% was used for base calling in the run summary.

To avoid sample DNA contamination, pre and post PCR were done on a separate workbench. A 0.5% sodium hypochlorite (10% bleach) was used to daily clean the workbench.

2.9 Variants calling analysis

Data from the MiSeq machine were in the FASTQ file format and were used to call the variant as specified in the flow chart below (Fig. 2.4).

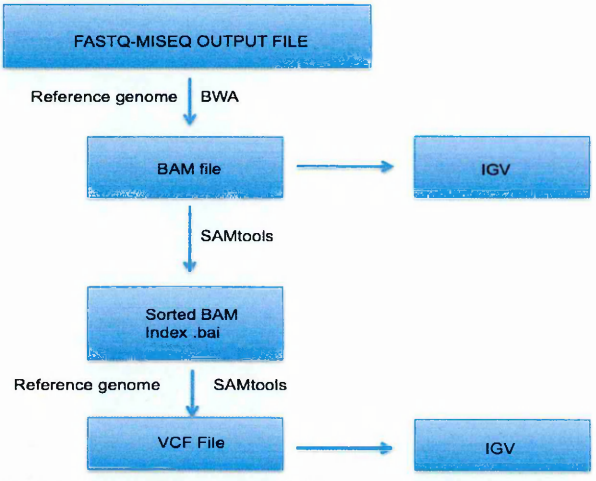


Figure 2.4 Flow chart of DNA sequence data analysis

The FASTQ output files from the illumina MiSeq machine are aligned to the reference genome using BWA to produce an output .BAM file. The SAMTOOLs uses the .BAM output file and reference genome to produce the .VCF text formats that contain the genotypes used for downstream data analysis.

Briefly, the FASTQ output file from the paired-end sequencing on MiSeq was aligned to chromosome (CHRM) 16: 72083574-72095400 on Hg19 using the Burrows-Wheeler Alignment tool (BWA). The BWA produced an alignment output in the BAM format, which showed reads aligned to the reference genome. Reads in the BAM format were in the same arrangement as the FASTQ, a line for a read.

The SAMtools was used for finding variants based on coverage (read depth), number of reads containing reference and alternate bases, mapping quality of the reads, and the existence of nearby indels. The SAMtools produced an ouput file called the sorted BAM format, an indexed binary (index .bai) file that is smaller, faster and occupies less space than the SAM format and also arranges the sequences by coordinates.

The reference genome and an appropriate SAMtools command line was used to produce the .VCF file containing the variants. A threshold of 20 for quality

score and 200 reads was used as the standard for variant calling.

For visualization, both the VCF and the BAM files were uploaded into the IGV and the appropriate coordinate for the gene was specified.

A control measure was introduced right from the outset to check for cross-contamination at both the amplicon and the library preparation; samples from mother, father, and children introduced into the assay were used as the control and the result indicated that there was no sample cross-contamination during the amplicon and library preparation. This is because the SNPs in the children were from either of the parents or both when visualized in the IGV. It would be highly unlikely for the SNPs found in the children samples to be identical to both or either of the parents if the samples were cross contaminated.

2.9.1 Detecting Known Variant from unknown Variant

The variation viewer in NCBI (http://www.ncbi.nlm.nih.gov/variation/view/) was used to search for known and unknown variants in the VCF file. The VCF file was uploaded to the variation viewer by clicking on the link, 'your data' at the search component. On uploading the VCF file, the coordinate of interest on the chromosomes was specified. The sequence viewer then displays the gene tract centered on the *Hp* gene with that of ClinVar short variation, ClinVar large variation and dbSNPs databases. The graphical display showed the uploaded data as added tracts (Fig.2.5). Clicking on the specified variant zoomed the display to the location of that variant/feature on the gene.

If the specified variants are known and already present in the database, there will be an alignment at the same position between those variant in the database and those in the added tract (Fig. 2.5). Unknown variants are found at a different position with no alignment to any variant in the databases (Fig. 2.5).

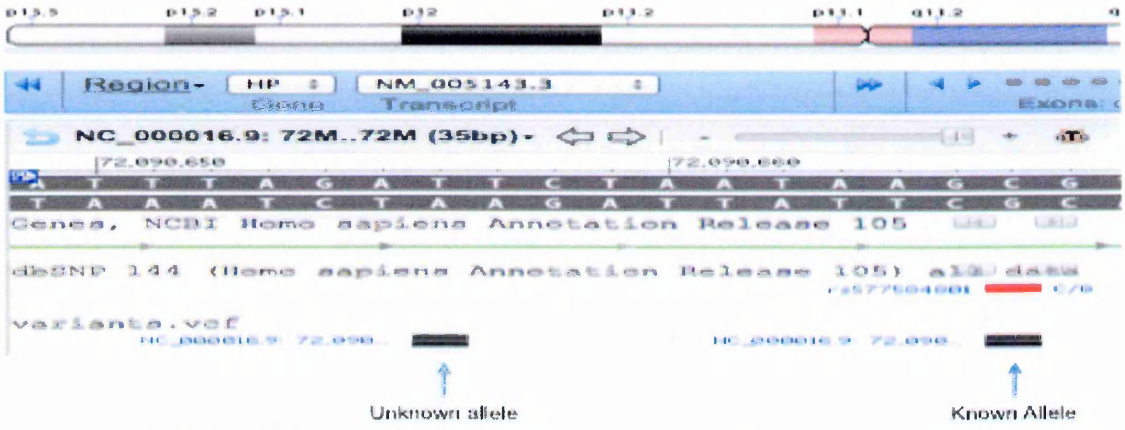


Figure 2.5 Identification of known and unknown Hp sequence variants

Any known variant in the uploaded .vcf file will align with the dbSNP variant, indicated in red colour and having a unique rs number (SNP ID). Unknown variant have no corresponding variant in dbSNP. Hp sequence corresponding to the position and region on the Hp gene where the variants occur are as displayed.

9.2 Plasma Hp levels and *Hp* gene sequence variants

To observe the cause and effect between plasma Hp levels and *Hp* gene sequence variants, the plasma Hp level of healthy individuals were studied in association with the sequence variants. An *Hp* gene variant was associated with plasma Hp levels by considering the population with the variants and having the corresponding plasma Hp level. An Hp variant was said to be associated with normal Hp plasma levels if >70% of the population with the variant have normal Hp plasma levels and *vice versa*. The association of the variants within the disease groups were then computed to determine the association of the variant with the disease group. Plasma Hp levels was considered independent of age and sex. There are no significant association between plasma Hp levels and age or sex.

CHAPTER THREE

3.0 DEMOGRAPHICS, STUDY DESIGN AND STATISTICAL ANALYSIS

3.1 Background

This chapter addresses the clinical and demographic data for the cohort as well as the preliminary investigations that form the basis of the research focus. This study was part of a research cluster under the auspices of the Childhood Malaria Research Group (CMRG) formed between the laboratory of Delmiro Fernandez-Reyes at the National Institute for Medical Research; now the Francis Crick Institute, UK and University College Hospital (UCH), Ibadan, Nigeria. Members of the cluster participated in clinical examination of the patients as well as the sample collection.

Trained physicians and laboratory scientists at the UCH, Ibadan collected both the clinical and demographic data. Both Florence Burte and I carried out the preliminary investigation and I performed all of the statistical analysis and interpretation of the demographic, cohort and preliminary data.

3.2 Ethical Statement

The ethical committee at the Institute for Advanced Medical Research and Training (IMRAT) of the College of Medicine, UCH, Ibadan gave approval for this research with permit number: UI/EC/10/0130. Parents or guardians of study participants gave written consent in accordance with the World Medical Association ethical principles for research involving human subjects.

3.3 Study site

All participants in the cohort were recruited from Ibadan, the largest city in sub-Saharan Africa with a dense population where malaria is holoendemic. All

study participants were recruited under the auspices of CMRG at the UCH, in the city of Ibadan, Nigeria.

The city has a lengthy 8-month rainy season, with more than 10 rainy days per month between May and October. However, there is scant rainfall between November and April, a period that includes the Harmattan characterized by a hot, dry and dusty northeasterly wind. Malaria transmission and severe disease occur throughout the year. More than 12,000 children visiting the hospital are diagnosed as malaria positive each year; SMA and CM account for 11.3 and 19.7% of the clinical cases, respectively. Although severe malaria syndromes are predominant in children under 5 years-of-age, there is a significantly large burden of severe disease in children up to 16 years-of-age (Orimadegun et al., 2007).

3.4 Subjects and case definitions

We recruited children aged between 6 and 165 months (Table 3.1) from 2010 to 2012. Three to four physicians examined each patient before the malaria positive clinical cases were defined. We define or categorize the malaria positive cases as the Uncomplicated Malaria (UM), Severe Malaria (SEVM), Severe Malarial Anaemia (SMA), and Cerebral Malaria (CM).

CM cases were defined as children with unarousable coma that persisted for more than one hour with generalized convulsion and the presence of asexual forms of *P. falciparum*. A Blantyre score of <2 was used to define coma status.

SMA was defined for conscious children with a packed cell volume (PCV) of <15% or a haemoglobin concentration <50g/L and the presence of asexual forms of the parasite and no other evident cause of the anaemia.

UM cases were defined as patients with fever and *P. falciparum* parasitaemia who did not require hospital admission and were recruited as part of a routine malaria parasite screening at the Children's Out-patient Clinic.

Patients with other complications of severe malaria such as respiratory distress, lactic acidosis, renal failure, hypoglycemia, and haemoglobinuria, and are without the strict definition of SMA and CM were regarded as severe malaria (SEVM) cases.

Bacterial blood culture positive cases and septicaemic patients with no manifestation of *P. falciparum* were excluded from the malaria groups but are included in the disease control; DC group. Patients with thalassaemia, sickle cell anaemia or G6PD deficiency were excluded from this study. The 1% of patients in the SMA and 10% of patients in the CM groups that died were excluded from the study. All participants in this study were effectively treated.

The disease control group (DC) comprised of malaria parasite-negative children with other forms of severe anaemia and fever of bacterial etiologies recruited on admission at the Otunba Tunwase Children's Emergency Ward.

The community control (CC) group consisted of malaria parasite-negative healthy children who were recruited on school visits within the city of Ibadan.

Terms such as malaria groups are used to represent all the clinical malaria groups; UM, SEVM, CM and SMA. The severe groups represent the SMA, CM and SEVM. The disease group represents the entire malaria group and the DC while the clinical groups represent all the malaria groups and the controls, the CC and the DC.

3.5 Sample collection

Plasma samples were collected at hospital admission and through convalescence. The first sample collected on the day of admission prior to drug treatment and/or transfusions in the case of SMA patients were labeled as plasma sample day zero (PD0). Plasma samples collected at day 7 and 14 were designated as plasma sample day 7 (PD7) and day 14 (PD14). We collected other

samples after the patient has been certified as fully recovered by a trained physician not part of this study. These samples were collected varying from day 28 to 50 and designated as PD28, since the majority was collected at day 28. Samples were collected at PD28 only after the subjects were certified as parasite free by microscopy.

A very short needle was used to collect the blood into the blood tube firmly screwed to the syringe. This was to avoid dispensing blood into the tubes through the syringe, which might cause haemolysis of some red blood cells.

The majority of the subjects were lost in the follow up (Table 3.1), however, those subjects that were lost within each disease group have similar clinical characteristics with those subjects that remain, hence the subjects lost in the follow-up are unlikely to influence the result.

3.6 Clinical analysis and limitations

Clinical data were collected using a malaria-tailored questionnaire designed by the CMRG. Blood samples were collected in an EDTA blood tube and transferred on ice to the central malaria laboratory. Plasma was harvested following centrifugation (1000*g*, 10 minutes) and aliquots were frozen at −80°C no later than 4 hours following collection.

Packed cell volume (PCV) was measured using the microhaematocrit method. Briefly, blood was collected in a capillary tube and was centrifuged at 12,000*g* for 5 minutes. The cell volume as a percentage of the whole tube volume was calculated.

Malaria parasites were detected and counted by microscopy following Giemsa staining of thick and thin blood films. Malaria Parasite (MP) densities were calculated as: MP/μL = [(number malaria parasites/number white blood cells) x 8,000]. The microscopy criterion for a participant to be free of malaria was the

absence of parasites in 100 high-power (1000X) fields. For validation and quality control, 1 in 10 thick blood films was randomly selected and examined independently by a local experienced microscopist not part of the research team. The Haemoglobinopathy screening and blood cultures were performed by HPLC and blood cultures respectively by scientist not part of this study. Subjects with any Haemoglobinopathy disorder and those with positive blood cultures were excluded from the malaria groups. Data for lactate/renal functions and glucose level were not made available but seems to have been determined locally in Ibadan for those in the SEVM group.

Quinine was administered intravenously to the entire group of patients in the CM group while quinine or artemether and lumefantrine under the trade name coartem in Nigeria were mostly used for subjects in other malaria groups. The entire SMA subject group was transfused at admission to the hospital after sample collection at PD0.

Certain parameters such as the Full Blood Count (FBC) including the Mean Corpuscular Volume (MCV), Mean Corpuscular Haemoglobin (MCH) and mean Corpuscular Haemoglobin Concentration (MCHC) were not available. The reticulocyte counts in each disease groups were not consistently recorded and are not reported in this study.

3.7 The cohort table

A total of 1, 622 plasma samples were collected at PD0, including samples collected for the DC and CC groups (Table 3.1). Subsequently, the numbers of samples decrease with follow-up to 249, 238 and 222 at PD7, 14 and 28 respectively. Hence, full complement of samples was not obtained for all patients; some patients were missed through the convalescence phase (Table 3.1). Single time-point samples were collected for both the DC and the CC group.

The age distributions in months as well as the PCV are presented as median with interquartile range; the lowest median age was that of the SMA and SEVM groups (Table 3.1). Low PCV was measured in the SMA, the CM+SMA groups and the DC group with severe non-malarial anaemia (Table 3.1). The DC group consisted of those with severe non-malarial anaemia with PCV value of 14(8-23) and disease of other etiologies with PCV value of 32(27-35) (Table 3.1).

Though there was no significant difference ($p=0.0657$) in the PD among groups, a more detailed F-test showed slight differences in their mean values, $F=2.219$. The PD in the SMA is higher than those of other severe groups (Table 3.1). The Mann Whitney test showed that the PD in the SMA, CM and SEVM were statistically higher ($P<0.05$) than the UM.

Another study on this same cohort, measuring the plasma level of pfHRP2 as an index of parasite biomass showed that the levels of pfHRP2 were significantly higher in the SMA group than the UM group (Huang et al., 2015), which was similar to the observed PD in the cohort. There were no differences in the PD when adjusted for age in both the severe and the uncomplicated malaria groups (data not shown). Malaria parasites were not detected in the DC and CC groups (Table 3.1). Individuals with overlapping CM and SMA syndromes (CM+SMA) are of lower age group (Table 3.1). Females and males were equally represented in the study (Table 3.1).

Table 3.1 The General cohort table

COHORT INFO	Groups						
	SMA	CM	CM + SMA	SEVM	UM	DC	CC
PD0 (N)	119	102	38	102	284	74	194
PD7 (N)	60	65	0	46	78	N/A	N/A
PD14 (N)	60	63	0	38	77	N/A	N/A
PD28 (N)	59	56	0	34	73	N/A	N/A
Age, months: M (IQR)	37 (6-150)	49 (15-165)	33(10-75)	37 (6-155)	45 (6-145)	55 (5-150)	50 (10-100)
Sex: F/M	55/64	50/52	20/18	48/54	140/144	39/35	99/95
PD: Mean (range)	87002[§] (11327-210275)	86559[§] (10240-191133)	71504 (2318-151692)	86419[§] (10455-199323)	64907 (3647-170348)	-ve	-ve
PCV: M (IQR)	13 (5-21)****	26 (17-40)*	13(12-16) ****	25 (15-38)	32 (16-42)[$$$$]	14 (8-23)[X]***** and 32(27-35)[f]	35 (27-40)
DNA sample sequenced	82	66	37	24	158	55	99

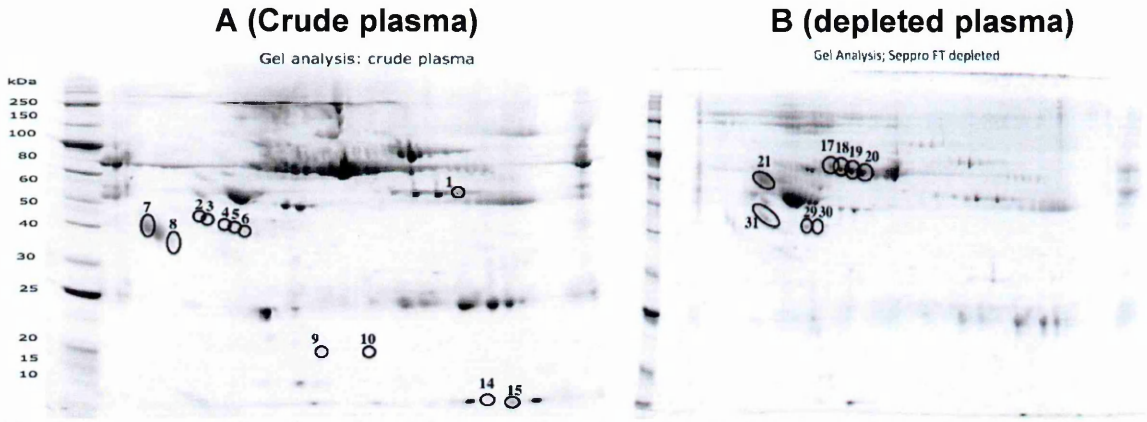
PD0, PD7, PD14 and PD28 are plasma samples collected at day 0, 7, 14 and 28 respectively; Severe Malarial Anaemia (SMA); Cerebral Malaria (CM); Severe Malaria (SEVM); Uncomplicated Malaria (UM); Disease Control (DC); Community Controls (CC); N= Number; PD = Parasite Density; PCV = Packed Cell Volume; M = Median. (IQR) = interquartile range; N/A = Not Applicable; -ve = Negative; ****Statistically lower than CM and SEVM values; *Statistically higher than SEVM value; [$$$$]Statistically higher than CM, SEVM, SMA and DC values; [§]Statistically higher to UM. The PD in the entire severe malaria groups was not statistically different to one another but different to PD in the uncomplicated malaria (UM). [f]PCV from DC group that comprises of those with fever of bacterial aetiology; [X]PCV from DC group that comprises of those with severe anaemia of non-malaria aetiology. The disease group is subdivided into two groups based on the PCV level; those with low PCV and are severely anaemic, which are used as the disease controls (DC) in chapter 4 and those with normal PCV and mostly consists of subjects with typhoid fever and other bacterial infections. Samples in both the DC and CC groups were only collected at PD0. The samples used in the preliminary studies are a pool of 30 samples randomly drawn from the clinical groups (CM, SMA, CM, UM) at PD0. While the preliminary studies were performed on both crude and immunodepleted samples, the data presented in the result chapter 4, 5 and 7 aside the genetic data in chapter 6 are based on individual crude samples. The number of DNA samples sequenced per disease groups are as indicated.

3.81 Preliminary observation and study design

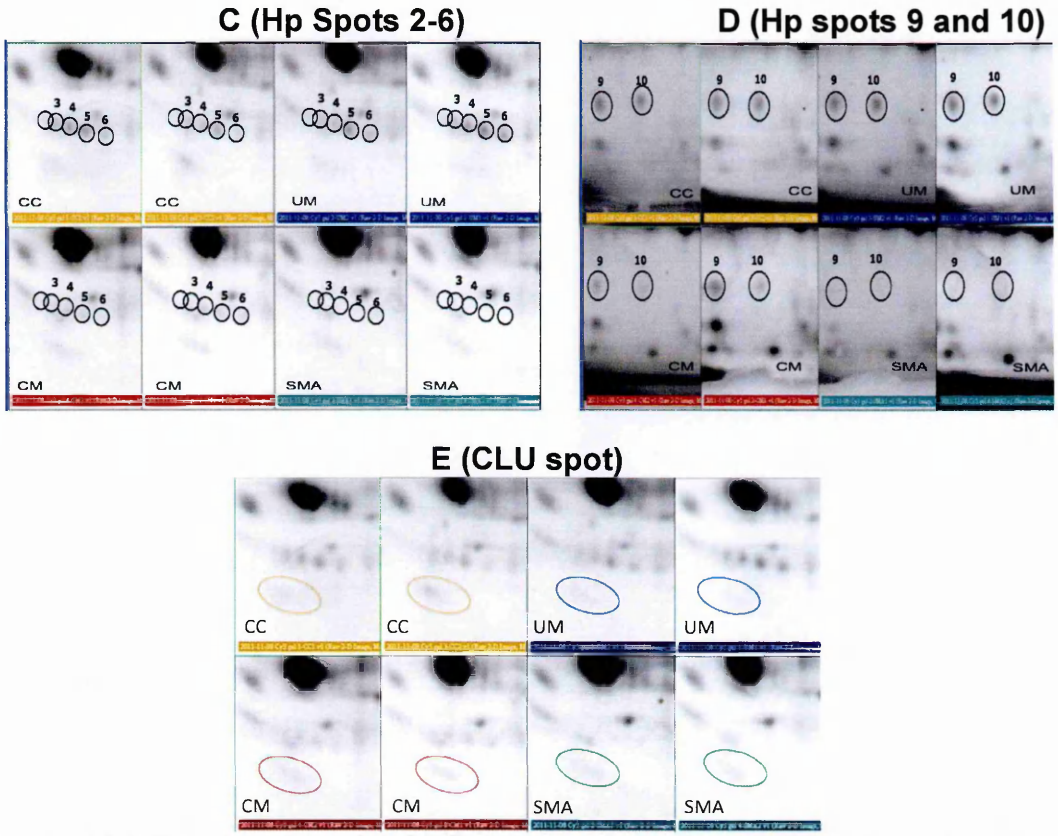
The major observation that formed the basis for this study was that the levels of Hp (both the β and α-chains) were as depleted in the CM as in the SMA (Fig. 3.1 C-D). Though there is some degree of haemolysis in the CM group, it is not known to be as severe as that of the SMA. We were therefore interested in why the plasma Hp level in the CM group is as depleted as that of the SMA? We then hypothesized that it is either Hp is congenitally low or has some yet unknown function in malaria syndromes that is responsible for its depleted level. To investigate our hypothesis, Hp levels were measured in the individual crude samples at acute onset and through convalescence to see if the low levels at acute onset will recover to normal on patients' recovery. The basis for our hypothesis was that low Hp levels as the consequence of the disease will returns to normal on patients' recovery after the disease causing haemolysis has been treated.

The levels of plasma HPx and some relevant proteins involved in malaria associated intravascular haemolysis were also quantified at acute onset and through convalescence to recovery. Plasma CLU levels were also quantified. Aside the preliminary experiments with 30 pooled samples; all other further investigations were based on crude plasma from individual samples.

Figure 3.1 Protein spots identified on A) Crude plasma; B) Immunodepleted plasma; C) Hp spots; D) Hp Alpha-2 spots; E) Clusterine spots



Numbers represent location of protein spots; Spot 1 is α1-anti-chymotrypsin spot, spots 2-6 are haptoglobin spots, spots 7 and 8 are α1-acid-glycoprotein 1 and 2 respectively, spots 9 and 10 are haptoglobin (alpha chains spots); spots 14 and 15 are haemoglobin β and α, spots 17-20 are haemopexin spots, spot 29 and 30 are both apolipoprotein AIV, spot 31 is clusterin.
Only Fig. 3.1B was performed using immunodepleted crude sample to remove excess albumin to reveal hemopexin (HPx) at its proximity.



Figures. 3.1 C-E are two-dimensional-difference-gel-electrophoresis (2DIGE) gel pictures from crude plasma in the disease groups. In order to identify proteins that are depleted in the disease groups (Fig. 3.1C-E), the two-dimensional-gel-electrophoresis (2DE) images from the CC (Fig. 3.1A-B) were used to align with the 2DIGE images (Fig.3.1C-E) and corresponding spots on the 2D gel are excised for mass spectrometry identification.
These figures are based on 30 pool samples collected at acute onset (PD0).

Table 3.2 Mass spectrometry data for identified proteins

ID no.	Accession	Description	Score	Coverage	# Peptides	MW [kDa]	calc. pI
1, 21	P01011	Alpha-1-antichymotrypsin [AACT_HUMAN]	213	16.31%	6	47.6	5.5
31	P10909	Clusterin [CLUS_HUMAN]	209.83	10.91%	5	52.5	6.27
29, 30	P06727	Apolipoprotein A-IV [APOA4_HUMAN]	2498	63.64	35	45.4	5.4
17, 18, 19, 20	P02790	Hemopexin [HEMO_HUMAN]	203	29%	12	51.6	7.0
2, 3	P00738	Haptoglobin [HPT_HUMAN]	378	21.43%	8	45.2	6.6
4	P00738	Haptoglobin [HPT_HUMAN]	1214	35.22	16	45.2	6.6
5	P00738	Haptoglobin [HPT_HUMAN]	712	31.28	14	45.2	6.6
6	P00738	Haptoglobin [HPT_HUMAN]	378	21.43%	8	45.2	6.6
9	P00739	Haptoglobin [HPT_HUMAN]	339	16.75	8	45.2	6.6
10	P00740	Haptoglobin [HPT_HUMAN]	456	11.58	8	45.2	6.6
7	P19652	Alpha-1-acid glycoprotein 2 [A1AG2_HUMAN]	171	25.87	7	23.6	5.1
8	P02764	Alpha-1-acid glycoprotein 1 [A1AG1_HUMAN]	822	33.83	8	23.5	5.0
14	P68871	Hemoglobin subunit beta [HBB_HUMAN]	290	55.78%	7	16.0	7.3
15	P01935	Hemoglobin subunit alpha [HBA_HUMAN]	345	39.72%	5	15.2	8.7

Each spot was identified by mass spectrometry, which shows the number of peptides found in each spot and the percentage coverage relative to the entire protein. Each spot is assigned a score that was used to identify the protein, which has a unique accession number and name in the database (Mascot database). The molecular weight (MW) and the Isoelectric point (pI) for each protein spot on the gel are as shown in the table. Protein spots with similar scores are written together as with HPx spot 17, 18, 19 and 20 while spots with different scores are shown differently in each row as with Hp.

Table 3.3 Differentially expressed protein on 2DIGE

Spot	UM	SMA	CM	Protein identification	Gene name	Gel analysis
Acute phase proteins						
1	↑ 358%	↑ 537%	↑ 448%	Alpha-1-antichymotrypsin	SERPINA3	crude plasma
21	↑ 103%	↑ 256%	↑ 249%	Alpha-1-antichymotrypsin	SERPINA3	Immunodepleted
8	↑ 296%	↑ 572%	↑ 906%	Alpha-1-acid glycoprotein 1/2	ORM1/2	crude plasma
7	↑ 68%	↑ 257%	↑ 282%	Alpha-1-acid glycoprotein 1	ORM1	crude plasma
Cholesterol transport						
29	↓ 95%	↑ 268%	↑ 132%	Apolipoprotein AIV	APOA4	Immunodepleted
30	↓ 69%	↑ 265%	↑ 44%	Apolipoprotein AIV	APOA4	Immunodepleted
Heme-induced oxidative stress						
2, 3	↓ 18%	↓ 467%	↓ 37%	Haptoglobin-beta chain	HP	crude plasma
4	↑ 44%	↓↓↓	↓ 7%	Haptoglobin-beta chain	HP	crude plasma
5	↑ 41%	↓ 286%	↑ 8%	Haptoglobin-beta chain	HP	crude plasma
6	↑ 77%	↓ 208%	↑ 40%	Haptoglobin-beta chain	HP	crude plasma
9	↑ 53%	↓↓↓	↓ 100%	Haptoglobin-alpha-2-chain	HP	crude plasma
10	↑ 77%	↓↓↓	↓ 611%	Haptoglobin-alpha-2-chain	HP	crude plasma
17	↓ 25%	↓ 348%	↓ 111%	Hemopexin	HPX	Immunodepleted
18	↓ 40%	↓ 156%	↓ 137%	Hemopexin	HPX	Immunodepleted
19	↓ 56%	↓ 340%	↓ 115%	Hemopexin	HPX	Immunodepleted
20	↓ 41%	↓ 194%	↓ 124%	Hemopexin	HPX	Immunodepleted
14	↓ 77%	↑ 254%	↓ 217%	Hemoglobin subunit-beta	HBB	crude plasma
15	↑ 33%	↑ 764%	↑ 219%	Hemoglobin subunit-alpha	HBA1/2	crude plasma
Complement-oxidative stress						
31	↓ 51%	↓53%	↓ 155%	Clusterin	CLU	Immunodepleted

The spots intensities on 2DIGE gel were quantified relative to the CC in an arbitrary unit using the PDQUEST (Bio-rad) software. This result was based on 30 pool samples from each malaria groups at PD0. Samples from the DC and the SEVM were excluded from the preliminary studies.

Red arrows indicate more than 100% increase relative to CC, green indicate more than 100% decrease relative to CC. Black downward or upward arrows indicate less than 100% decrease or increase relative to CC. 3 green downward arrows indicate that the spot had completely disappeared. Absence of arrow indicates no difference to CC. Numbers correspond to spots positions as identified on 2DE gel as shown in table 3.2.

3.9 Statistical Analysis

Power statistic was used to determine the minimum sample size required in order to detect at least 20% difference with 80% power and a conventional level of alpha, 0.05. This standard convention was known to reduce the likelihood of making type 1 and 2 errors or detecting an effect in the absence of any such effect and vice *versa* (Bartko et al., 1988, Pulver et al., 1988, Sharp and Gahlinger, 1988).

The effect sizes (d or F) were computed along-side the p-values in order to know the actual size (magnitude) of the statistical difference between/among groups as measured by the p-values.

An online computation software (http://www.psychometrica.de/effect_size.html#fvalue), the mean, the Standard Deviation (SD) and the sample size were used in computing the d-values. The d-values were interpreted in accordance with Cohen's *d* guidelines (Cohen, 1988). The F-value detects difference in the mean values. Computed mean values were used for the F-test whether or not the mean values are shown in the charts. Outliers are not included in the computation of mean. The mean values were often computed using the column statistics feature of the prism software version 6.0 (v6.0). Generally, data are presented as median and interquartile range in a 10-90 percentile box plots, point graphs, scatter plots, tables and heat maps. Only the mean values were used in both the point graphs and the heatmaps. The prism v6.0 and the Excel spreadsheet were often used for the statistical analysis.

A one-way analysis of variance (ANOVA) and multiple comparison testing were performed to determine the difference among groups. ANOVA was not used if the computed Bartlett's test indicated that the ANOVA assumption for homogeneity of variance have been violated and in such cases, the Brown-Forsythe tests were performed in place of ANOVA. In computing for ANOVA, the

mean of each group were compared with the mean of every other group (multiple comparison testing). Outliers were not included during the computation of the mean as mentioned earlier; this was to avoid bias in the mean value. An unpaired nonparametric Mann-Whitney test was used to compare data between two non-paired groups while a paired nonparametric Wilcoxon test was used for paired data within each disease group such as paired PD0 and PD28 data. For results presented in the table format, nonparametric Spearman correlations to determine the 95% Confidence Interval (CI) and/or P-value at 0.05 were performed. Statistical analysis was adjusted for age, sex and PD in some cases to test for such likely factors that could introduce variations.

Chi-square tests were performed to determine the difference between the observed and the expected Hp frequencies and whether the observed Hp frequencies in the populations were at Hardy-Weinberg equilibrium or a deviation from the null hypothesis.

Receiver Operator Characteristic (ROC) curves was plotted with CLU data in chapter 7 to determine the specificity and the sensitivity of the CLU assay in discriminating among the clinical malaria syndromes; the probability of a positive test result in the presence of the disease and the probability of a positive test result in the absence of the disease was computed by plotting sensitivity against 1-specificity in a ROC curve and the curves were interpreted based on the area under the curve (area) and the p-values. The $p<0.001$ was considered to be significant.

Basic descriptive statistics such as mean, median, mode, ratio and frequencies were also used when necessary.

The analysis of genomic data was performed with Burrows-Wheeler's Aligner (BWA) and SAMtooLs. Both the .BAM and .VCF output files were viewed

in the Integrative Genome Viewer (IGV) as earlier mentioned in Chapter 2. The .VCF file was for further computation.

We computed an Odds Ratio (OR) and 95% Confidence Interval (CI) at the conventional level of alpha (0.05) in order to determine how strongly the presence or absence of a variant (SNP, indel) is associated with the disease in the population. Briefly, The OR is computed as

$$OR = \frac{a/b}{c/d}$$
$$= \frac{a \times d}{b \times c}$$

Where a = Number of the diseased group with the variant

b= Number of the Control group with the variant

c = Number of the diseased group without the variant

d = Number of the control group without the variant

These values were imputed into the MedCalc online computation software (https://www.medcalc.org/calc/odds_ratio.php) for the computation of the OR, CI and p-Values.

CHAPTER FOUR: RESULT 1

4.0 LOW Hp LEVELS COULD BE A RISK FACTOR FOR SMA

4.1 Background:

As discussed in the Introduction, Haptoglobin (Hp) being a scavenger of free-haemoglobin (fHb) is generally known to be a marker of haemolysis. Childhood *Plasmodium falciparum* Severe Malarial Anaemia (SMA) exhibits both haemolytic and inflammatory pathology.

Studies on hypohaptoglobinaemia (low plasma Hp) provide conflicting views regarding its aetiology in childhood malaria. On the one hand, the low level of plasma Hp associated with malaria infection was proposed to have a genetic cause, while on the other hand it has been attributed to the disease causing intravascular haemolysis (Delanghe et al., 1998c, Koda et al., 2000, Koda et al., 1998b, Delanghe and Langlois, 2001); Low plasma Hp is also considered as a marker of haemolysis in the SMA group (Ouyang et al., 1998) but whether it is a risk factor or a consequence of this syndrome is unknown.

Differentiating between these etiological scenarios should provide insights into the role of Hp in the pathology of malaria. In order to ascertain this role, a case-control study with longitudinal follow-up to recovery of children was performed in the cohort (Table 3.1).

As earlier discussed in chapter three, the plasma samples were collected through convalescence in all the malaria groups (Table 3.1). Samples were collected at day of admission (PD0), and through convalescence; day 7 (PD7), day 14 (PD14) and day 28. These samples were assayed for Hp and known markers of haemolysis: free haemoglobin (fHb) and lactate dehydrogenase; LDH (Ballas, 2013, Shah et al., 2014, Kato et al., 2006, Tabbara, 1992). The levels in children with SMA were compared to children with other malaria syndromes, malaria

negative severe anaemia used here as the disease control (DC) and healthy malaria-negative community controls (CC).

The hypothesis to be tested was that a low Hp level as a consequence of haemolysis would return to normal (20-200mg/dL) through Hp production after the underlying haemolysis had been arrested.

Studies have shown that a haemoglobin (Hb) level as low as 5.0 g/dL in severe malaria anaemia returns to normal 2-3 weeks after commencement of treatment (Raffray et al., 2014); there was a significant decrease in the level of total plasma haem at day 28 compared to day zero (Cunnington et al., 2012); reports have shown that Hp levels returned to normal within 1-2 weeks (Echeverry et al., 2016) and both Hb level and bone marrow morphology returns to normal as the parasite disappears from the blood either by therapy or host mechanism (Abdalla, 1990) within 3 weeks in the convalescence phase (Phillips et al., 1986). In line with these findings and coupled with the clinical observations by pediatricians, the 28-50 days recovery time-points might be sufficient for the Hp level and other clinical parameters to normalize. Hence, protein levels at PD28-50 are considered as equivalent to normal prior to malaria onset and are comparable to those of malaria-negative healthy CC.

The results in this chapter show that the low circulatory plasma level of Hp observed during an acute life-threatening childhood SMA episode is not a consequence of haemolysis but a risk factor for acute or insidious onset of SMA.

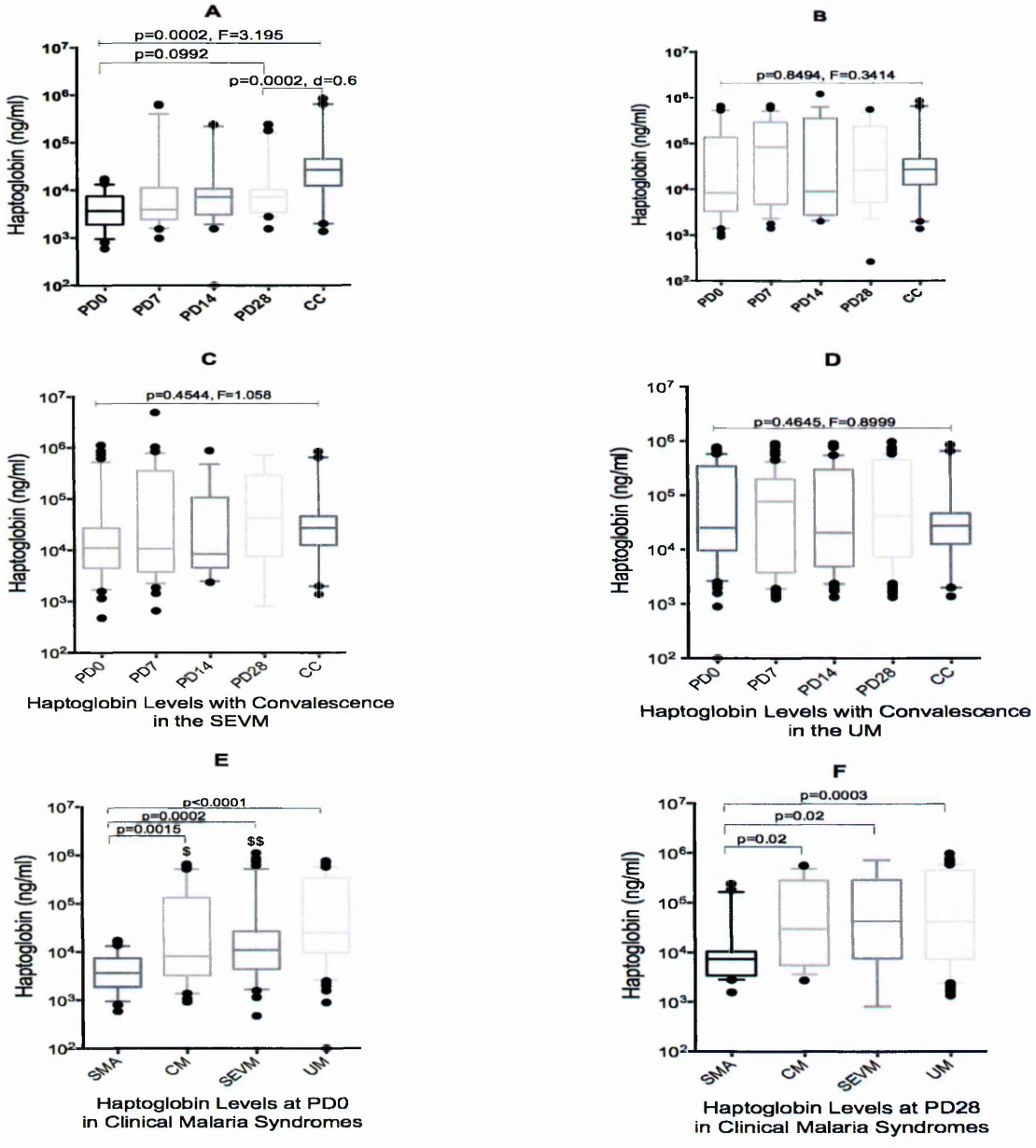
4.2 Plasma Hp level in the SMA group is stable through convalescence to full recovery

At time of diagnosis (PD0), Hp level is low in the SMA group and remains low all through convalescence to full recovery (Fig. 4.1A). Levels in the SMA group at recovery are significantly lower ($p=0.002$) compared to malaria negative healthy

CC (Fig. 4.1A). Further analysis shows that there is a large proportion of non-overlapping values; d=0.6 between Hp levels in the CC and that of the SMA at recovery and are not likely to be due to chance (Lakens, 2013, Cohen, 1988).

Unlike the observation with SMA patients, levels in the CM, SEVM and UM groups at recovery are not significantly different to the CC group (Fig. 4.1B-D). Levels of Hp among all the time-points within the CM, SEVM and UM groups are not statistically different except for slight variation in both the medians and the mean values (Fig. 4.1B-D). Levels of Hp in the SMA group are significantly lower at both onset and recovery compared to those of other malaria syndromes (Fig. 4.1E-F). The levels in both the CM and the SEVM groups are significantly lower than levels in the UM group at onset but not at recovery (Fig. 4.1E-F).

Figure 4.1 Hp levels with convalescence in the disease groups, A) SMA; B) CM; C) SEVM; D) UM and E) at PD0 and F) PD28

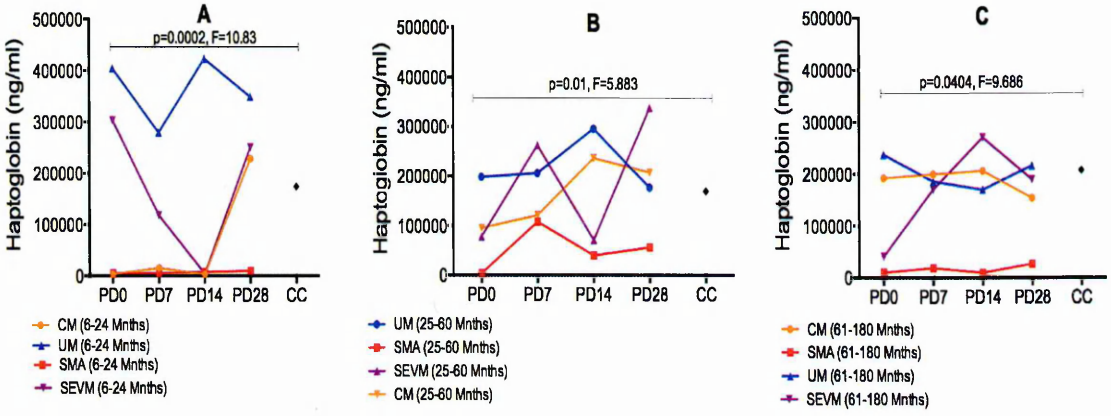


Graphs are presented as median interquartile range, 10-90 percentile. Outliers beyond 10-90 percentile are indicated as round black shapes on the graph. $^{\$}$Statistically different to UM ($, p=0.01; $$, p=0.009); p= p-value; F= fvalue and it indicates the differences in mean values among the time points, it's a measure of effect size. d= is also effect size but measures the difference between two time points, for example, d=0.6 as shown in Fig. 4.1A implies 60% non-overlapping values between SMA at PD0 and CC. The effect size shows the magnitude of the statistical difference as indicated by the p-value.

Figures 4.1 A-D are based on full complements of samples at all time points, PD0, PD7 and PD28 as shown in the general cohort table. Fig. 4.1 E and F are only at PD0 and PD28. Non-parametric Wilcoxon test was used to compare two groups while ANOVA (Tukey multiple comparison testing) was used for comparison among groups across the time points. $p<0.05$ was considered as statistically significant.

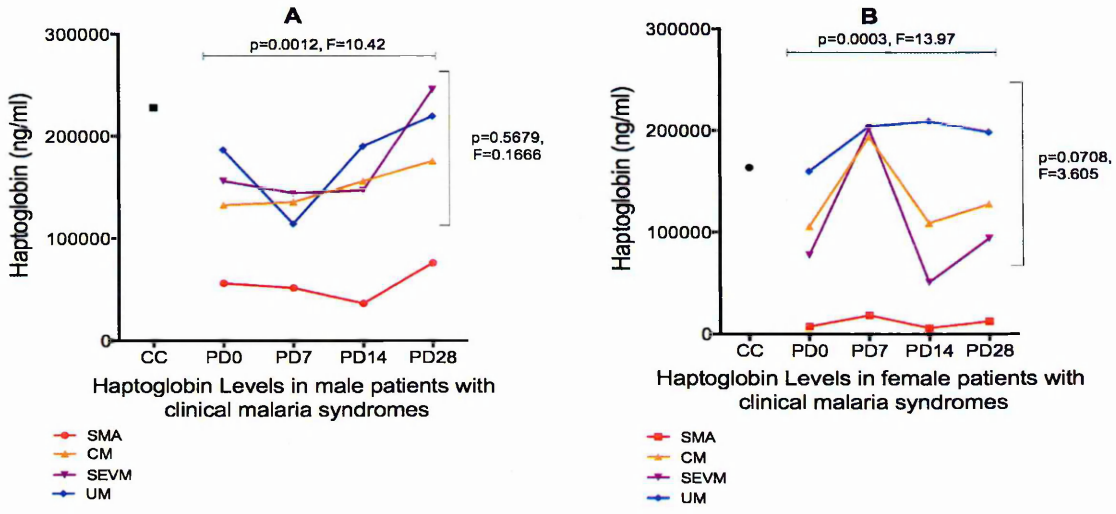
Figure 4.2 Mean Hp level with age, A) 6-24 months; B) 25-60 months;

C) 61-180 months



Mean Hp levels in subjects with full complements of samples (Table 3.1) are as presented on the graphs. Outliers were not included in the computation of these mean values. Non-parametric ANOVA was used for the statistical analysis and $p<0.05$ was considered as statistically significant. p=p-value and F=f-value.

Figure 4.3 Mean Hp levels with sex, A) Male; B) Female



Mean Hp levels in subjects with full complements of samples (Table 3.1) are as presented on the graphs. Outliers were not included in the computation of the mean values. Non-parametric ANOVA was used for the statistical analysis and $p<0.05$ was considered as statistically significant. p=p-value and F=f-value (effect size; differences in the mean values).

4.3 Hp levels in the SMA groups are independent of age, sex and parasite density

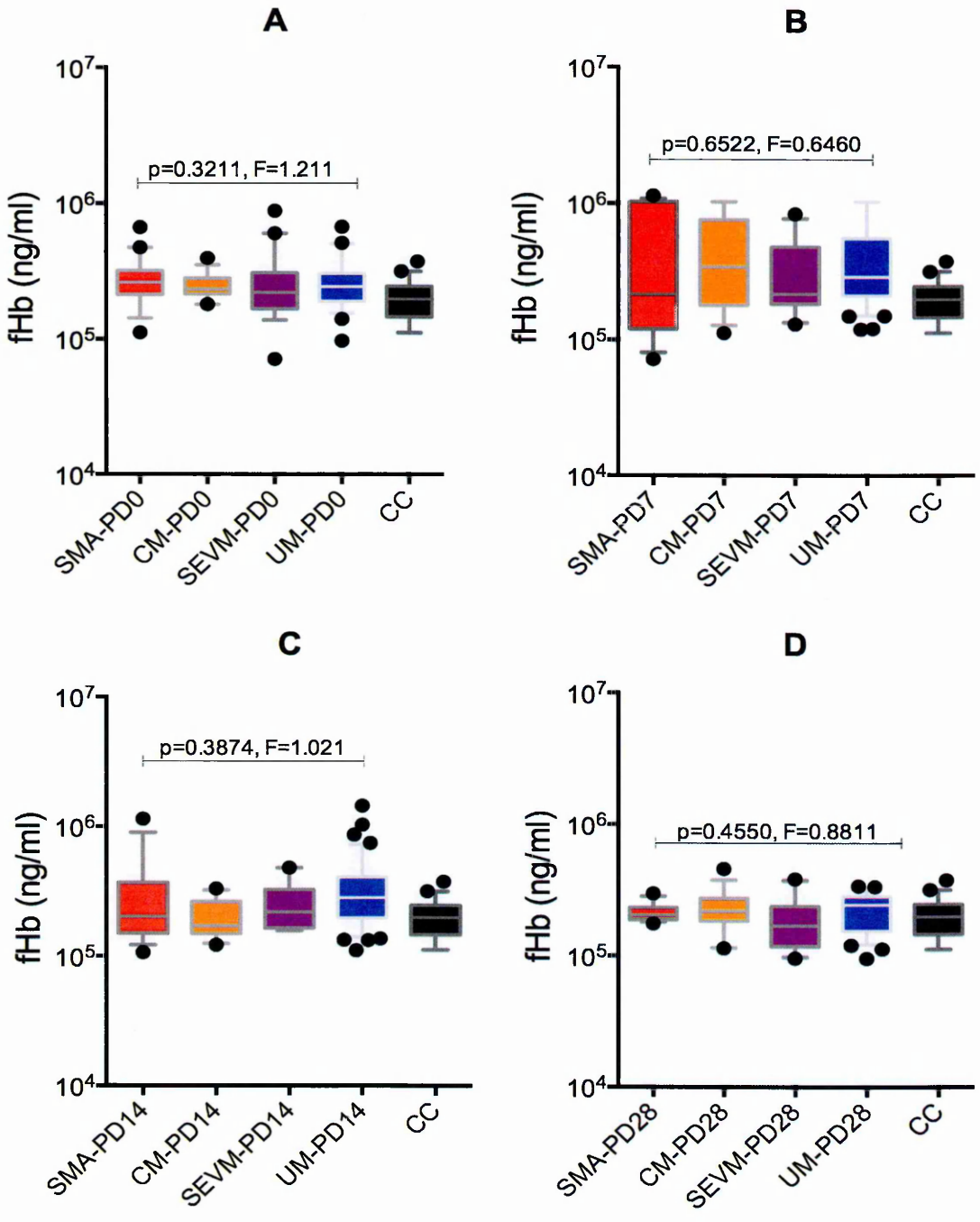
The levels of Hp in the SMA group remain lower in all age groups compared to other malaria and the CC groups (Fig. 4.2A-C). Unlike other malaria groups, levels of Hp in the SMA groups are stable across the age groups through convalescence, particularly in children at age 25-60 and 61-180 months (Fig. 4.2A-C). In the CM, SEVM and UM groups, the levels of Hp recover to normal with patients' recovery irrespective of both the age groups and the varying levels through convalescence (Fig. 4.2A-C).

The levels of Hp also remain low all through convalescence to recovery in both male and female subjects with SMA compared to the other malaria groups and the CC (Fig. 4.3A-B). Mean values of the circulatory Hp levels in male and female subjects with CM, SEVM and UM are not statistically different at recovery (Fig. 4.3A-B). The dynamics of Hp levels in the male and female subject are of different pattern (Figure 4.3A-B).

The significant difference observed in the entire malaria groups in both male ($p=0.0012$, $F=10.42$) and female ($p=0.0003$, $F=13.97$) subjects are influenced by the low plasma Hp level in the SMA group (Fig. 4.3A-B).

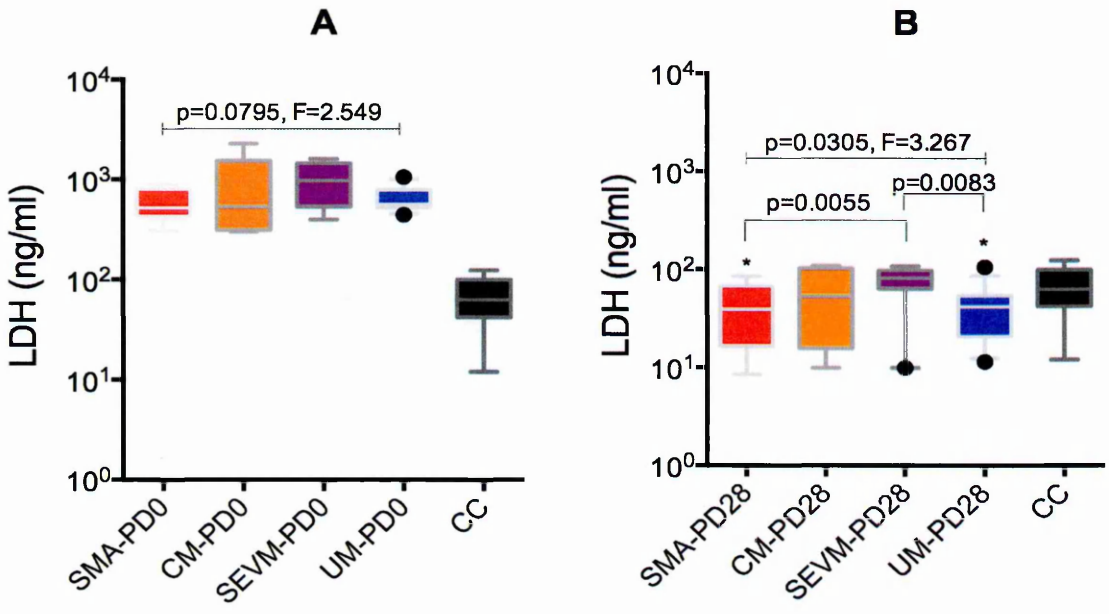
There is no significant correlation between the PD and the circulatory Hp level in the SMA group (Table 4.1b).

Figure 4.4 Free-Haemoglobin levels with convalescence across the malaria groups at A) PD0; B) PD7; C) PD14; D) PD28



Graphs are presented as median interquartile range, 10-90 percentile. Outliers beyond 10-90 percentile are indicated with round black dots on the graphs. Numbers within each disease groups at a given time point are as shown in the general cohort table 3.1. Non-parametric ANOVA was performed across the disease groups. $p<0.05$ was considered as being statistically significant.

Figure 4.5 Levels of LDH in the malarial groups at A) PD0 and B) PD28



Graphs are presented as median interquartile range, 10-90 percentile. Outliers beyond 10-90 percentile are indicated as round black dots on the graph. Numbers within each group are as shown in the general cohort table 3.1. Non-parametric ANOVA and Mann Whitney test were used to analyze the data. $p<0.05$ was considered as statistically significant. *=Statistically different to CC. The levels of LDH were measured only at both PD0 and PD28 in tandem with that of fHb as a second marker of haemolysis. LDH levels at PD0 agree with that of fHb at PD0 in that levels are not statistically different across the severe malaria groups and do indicate that haemolysis in the SMA is not higher than other severe malaria groups.

4.4 Plasma fHb and LDH levels in SMA are not different to other malaria groups

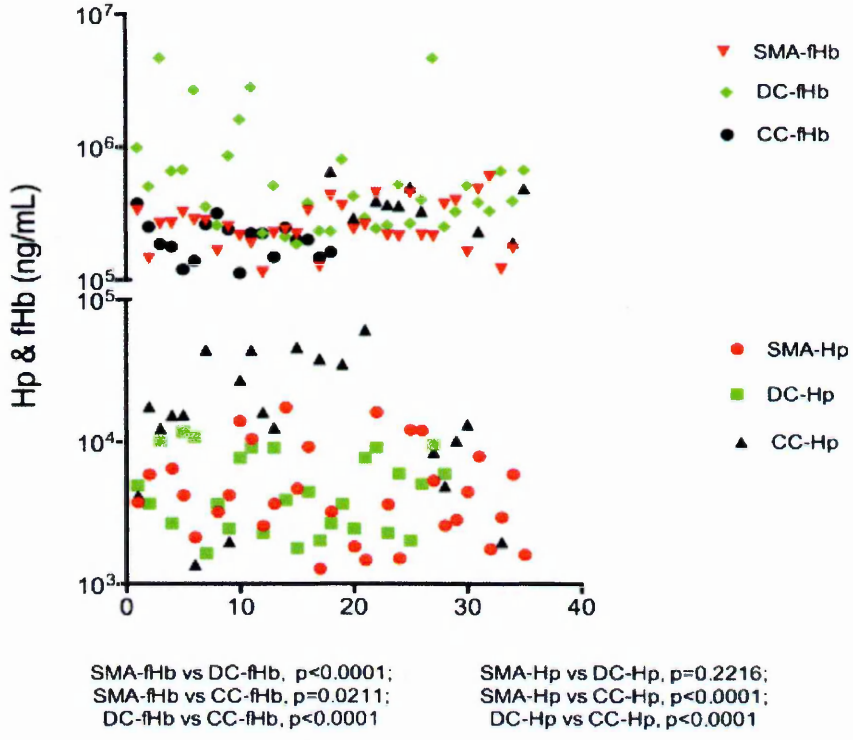
The levels of fHb at all the time points in the SMA group are not statistically different to that of other malaria groups (Fig. 4.4A-D). The levels of fHb in the entire disease groups are higher than those of CC at PD0 through PD14 but not at PD28 (Fig. 4.4A-D). The levels of fHb within the groups do not show the wide differences in levels as observed with Hp (Fig. 4.1A-F and 4.4A-D).

Levels of LDH are higher in the malaria groups than the CC group at onset. Levels among the malaria groups are not statistically different ($p=0.0795$, $F=2.549$), but the SEVM group shows a slightly higher median (Fig. 4.5A). At recovery, the levels of LDH in the malaria groups, except the SEVM group show lower median compared to that of the CC; the level in both the SMA and the UM are statistically lower than both the SEVM and the CC (Fig. 4.5B). While there are no differences in the plasma LDH levels between the malaria groups at onset, differences exist at recovery (Fig. 4.5A-B).

4.5 The levels of both fHb and Hp in the SMA behave differently to the DC; non-malaria severe anaemia.

While the levels of Hp in both the SMA and the DC groups are the same and are both lower than the level in the CC group at acute onset, the level of fHb in the DC group is significantly higher than the level in the SMA group (Fig. 4.6). There is a positive correlation between fHb and and Hp levels in the DC group (Table 4.2A).

Figure 4.6 Levels of both Hp and fHb in SMA and severely anaemic parasite negative DC group at PD0



The vertical axis indicates the concentrations of both fHb and Hp while the horizontal axis indicates the number of samples. Non-parametric Mann-Whitney test was used to make comparison between levels; such as level of fHb in the SMA versus the DC (SMA-fHb vs DC-fHb) as shown under the graph. This experiment was performed in order to check for the level of Hp and fHb in malaria negative severely anemic subjects (DC). A total of 35 samples were randomly picked from both the SMA and the DC groups at PD0; however, the numbers used in the computation are lower due to the removal of outliers, particularly from the Hp level in the DC group.

4.6 Plasma fHb but not Hp correlates with PD in children with SMA

While there is a significant positive correlation between fHb and PD in the SMA group, there is no significant correlation between fHb and PD in the rest of the malarial disease groups (Table 4.1.A).

The levels of Hp correlate negatively with parasite density only in the UM group (Table 4.1.b). There are significant negative correlation at both acute onset and at recovery between fHb and Hp in the SEVM group (Table 4.2.a-b) and a significant positive correlation between fHb and Hp in the DC group (Table4.2.a), as mentioned earlier.

Table 4.1 Correlation at PD0 between A) fHb level and parasite density; B) Hp level and parasite density

	A. fHb and Parasite Density at PD0			B. Hp and Parasite Density at PD0		
	r	*95% c.i.*	*p value*	*r*	*95% c.i.*	*p value*
UM	-0.1025	-0.4611 to 0.2849	0.2984	-0.2423	-0.4682 to 0.01339	0.0275*
SEVM	-0.1607	-0.5828 to 0.3297	0.2556	-0.1364	-0.4489 to 0.2059	0.2105
CM	0.1974	-0.3269 to 0.6288	0.2224	-0.2125	-0.5200 to 0.1457	0.1138
SMA	0.3829	-0.08593 to 0.7128	0.0300*	-0.1906	-0.5046 to 0.1680	0.2804

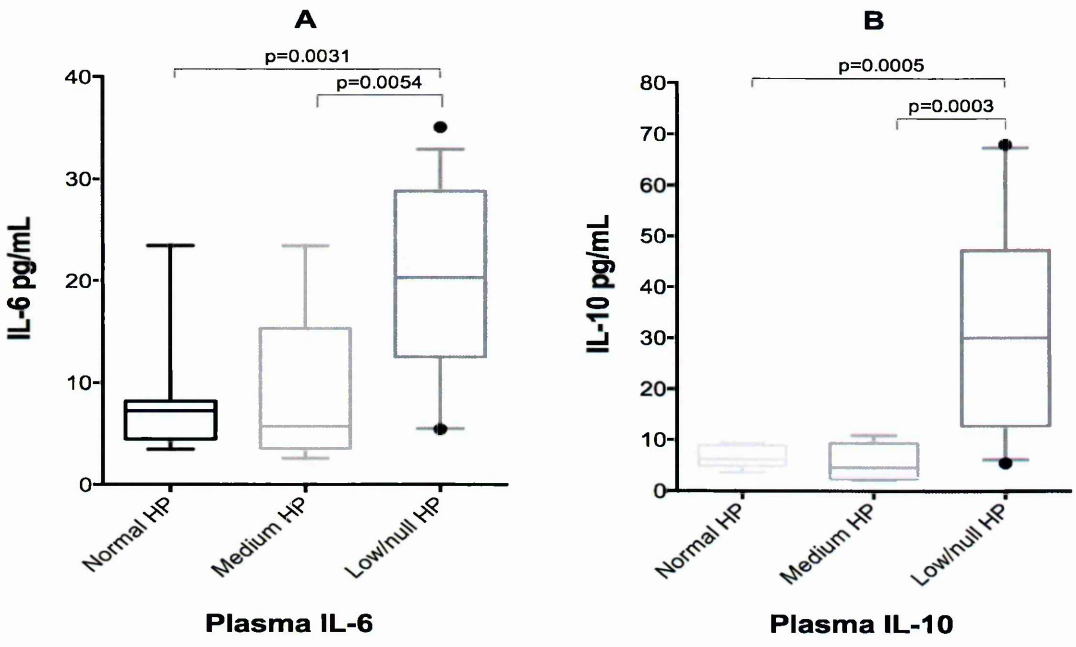
* = Statistically significant, P<0.05; c.i = Confidence interval; r = regression. Non-parametric Spearman correlation was used.

Table 4.2 Correlation between fHb and Hp at both A) PD0 and B) PD28

	A. Hp and fHb levels at recruitment (PD0)			B. Hp and fHb levels at recovery (PD28)		
	r	*95% c.i.*	*p value*	*r*	*95% c.i.*	*p value*
UM	0.1303	-0.2587 to 0.4830	0.2502	-0.1798	-0.4156 to 0.07865	0.0793
SEVM	-0.6003	-0.8331 to -0.1867	0.0033**	-0.4552	-0.7742 to 0.04815	0.0335*
CM	-0.02941	-0.5145 to 0.4700	0.4567	0.4000	-0.1577 to 0.7642	0.0704
SMA	0.1387	-0.3497 to 0.5677	0.2857	0.2672	-0.1373 to 0.5953	0.0889
DC	0.4737	0.01021 to 0.7696	0.0202*	N/A	N/A	N/A
CC	-0.08428	-0.5292 to 0.3970	0.3658	N/A	N/A	N/A

* = Statistically significant, P<0.05; c.i = Confidence interval; r = regression; N/A = Not Applicable.
Non-parametric Spearman correlation was used.

Figure 4.7; Levels of A) IL-6; and B) IL-10 in SMA subjects with normal, medium and null Hp levels at PD28.



Graphs are presented as median interquartile range with 10-90 percentile. Outliers beyond 10-90 percentile are indicated as round black dots on the graph. Non-parametric Mann-Whitney and ANOVA tests were used for the statistical analysis. The numbers (N) of subjects with low, medium and normal levels of Hp at PD28 are 20, 11 and 5 respectively. These subjects were randomly selected from the SMA group at PD28.

4.7 The low plasma Hp level group is associated with high IL-6 and IL-10 level

SMA subjects with low/null Hp level have significantly higher levels of both IL-6 and IL-10 compared to subjects with both normal and medium Hp levels (Fig. 4.7A-B). Both the levels of IL-6 and IL-10 are not statistically different in the subjects with both normal and medium Hp levels (Fig. 4.7 A-B).

4.9 Discussion

Since the discovery of Hp in 1938 (Wobeto et al., 2008b), its levels have been widely used as a marker of haemolysis (Gupta, 2011). However, genetic factors, inflammation and other yet unknown etiologies can also affect plasma levels of Hp (Koda et al., 1998b).

In the pathological context of clinical malaria, and in particular SMA, genetic associations, inflammatory responses and haemolysis-induced anaemia have been implicated (Casals-Pascual and Roberts, 2006, Koda et al., 1998a, Koda et al., 1998b), therefore the causes and consequences of low Hp plasma levels are difficult to dissect. A common assumption has been that low Hp levels in SMA patients result from parasite-induced haemolysis (Boreham et al., 1981, Langlois and Delanghe, 1996). However, by measuring the levels of Hp, fHb and LDH on hospital admission, during the convalescence and after recovery, we show that low Hp levels increase susceptibility to SMA, rather than being a result of the *P. falciparum* infection and its associated haemolysis.

In the present study, Hp levels on admission were lowest in children with SMA and comparable to those in the malaria-negative DC group, an observation that appears consistent with the haemolysis-induced hypohaptoglobinaemia hypothesis (Gupta et al., 2011, Imrie et al., 2006, Kim et al., 2012a, Delanghe et al., 1998b). Furthermore, the low level of Hp is not specifically restricted to children

with SMA as the levels at the time of admission and prior to any form of treatment were also low in all severe disease groups compared to the levels in the UM and CC groups. However, the Hp levels remained low at onset and during convalescence to full recovery in children presenting with SMA, suggesting that Hp levels were already low in these patients prior to the malaria episode and the onset of SMA. Therefore, the low and stable level of Hp in the SMA couldn't have been a consequence of the disease or higher intravascular haemolysis; our data show that though the level of fHb and LDH are significantly higher in children presenting with acute onset SMA than in malaria-negative CC, the levels of both fHb and LDH in the SMA group are not different to other severe malaria-positive groups contrary to the observation with malaria-negative severe anaemia; DC.

It would not have been possible to ascertain the aetiology of congenital hypohaptoglobinaemia if the level of Hp was measured only at the onset of malaria.

Although a/hypohaptoglobinaemia can be genetic in origin, pathological conditions associated with the disease can also be equally important; a null level of IL-6 suppresses Hp expression (Oliviero et al., 1987). But in this case, the level of IL-6, an inducer of Hp does not seem to be responsible for the low/null Hp levels in the population considering the higher IL-6 level that is associated with the low Hp group. We suggest that the low/null level of Hp through convalescence to full recovery in the SMA group is rather genetic in origin and is a risk factor for onset of SMA.

In acquired hypohaptoglobinaemia induced by malaria or by other haemolytic disease the level of Hp should return to normal after the commencement of treatment of the etiological cause (Saad and Geoffery, 2004, Trape et al., 1985, Rougemont et al., 1988). This study clearly differentiates acquired from congenital a/hypohaptoglobinaemia, which is worthy of note as

a/hypohaptoglobinaemia is being used as the metrics for measuring the efficacy of malarial prevention or control strategies (Imrie et al., 2012, Imrie et al., 2006).

We therefore suggest that while the majority of fHb in the SMA is linked to parasitic destruction of RBC, direct clearance of both infected and uninfected RBC by the spleen rather than intravascular haemolysis could be more responsible for the low haemoglobin (Hb) level in the SMA.

Our data revealed that at day-of-recruitment and at full-recovery, the level of LDH shows similar trend to the level of fHb in all the malaria-positive cohorts. These findings point to the importance of the fHb and LDH turnover dynamics in the establishment of the major clinical malaria syndromes.

Our study provides novel insights into the Hp-Hb balance in children presenting with acute or insidious onset of SMA. In this context, we show that Hp and fHb cannot be used as indicators of haemolysis severity. The circulatory markers of haemolysis in the anaemia of children with SMA may be strikingly different to those with anaemia of non-malaria aetiology. Most importantly, we show that low plasma Hp level in this population is a risk factor for the occurrence of SMA in contrast to the other malaria syndromes studied.

In the present study we show that the longitudinal measurement of Hp level during convalescence and full recovery is important to differentiate congenital from acquired hypohaptoglobinaemia. Furthermore, our findings provide evidence that the hypohaptoglobinaemia phenotype can be put forward as a predictor of whether or not a child with uncomplicated malaria will go onto developing an acute-onset SMA episode in the clinic.

CHAPTER FIVE: RESULT 2

5.0 DO HAPTOGLOBIN PHENOTYPES MODULATE RESPONSE TO MALARIA ILLNESS?

5.1 Background

As earlier mentioned, the 3 major Hp phenotypes show marked geographical distribution. These phenotypes have also been associated with susceptibility to and protection from diseases such as diabetes, heart disease, cancer, HIV as well as malaria. However, the association studies on Hp phenotypes and malaria are contradictory and based only on their frequencies in malaria within given phenotypes. In addition, it has been reported that the binding affinity of Hp to fHb and the rate of fHb clearance by Hp via CD163 receptors are dependent on Hp phenotypes (Kasvosve et al., 2010, Quaye, 2008, Kristiansen et al., 2001), indicating their possible role in disease.

The hypothesis/question to be addressed in this chapter is that, do individuals with different Hp phenotypes but presenting with the same malaria syndrome and *vice versa* mount the same response to the illness? Since plasma proteins are a good reflection of disease pathophysiology, plasma Hp levels and that of markers of oxidative stress, macrophage activation, cytokines and dyserythropoiesis were measured both at acute onset of the disease and at recovery in individuals with different Hp phenotypes. This is with the aim to study the possible effects of Hp phenotypes in the pathophysiology of the different malaria syndromes. Oxidative stress, cytokine imbalance and dyserythropoiesis were thought to be the underlying mechanisms behind SMA with no clear definition as to which is the major mechanism. The results here show that the plasma levels of SOD-1; a marker of oxidative stress, macrophage associated chemokines and both pro- and anti-inflammatory cytokines differ among individuals with different Hp phenotypes but presenting with the same malaria

syndrome as with different malaria syndromes but the same Hp phenotypes. The results spelt out here could possibly explain the differences or similarity in response to malaria illness and their possible associated pathophysiology among individuals. This chapter also reports on whether the association between malaria and Hp phenotype frequencies are restricted to malaria or present in other disease.

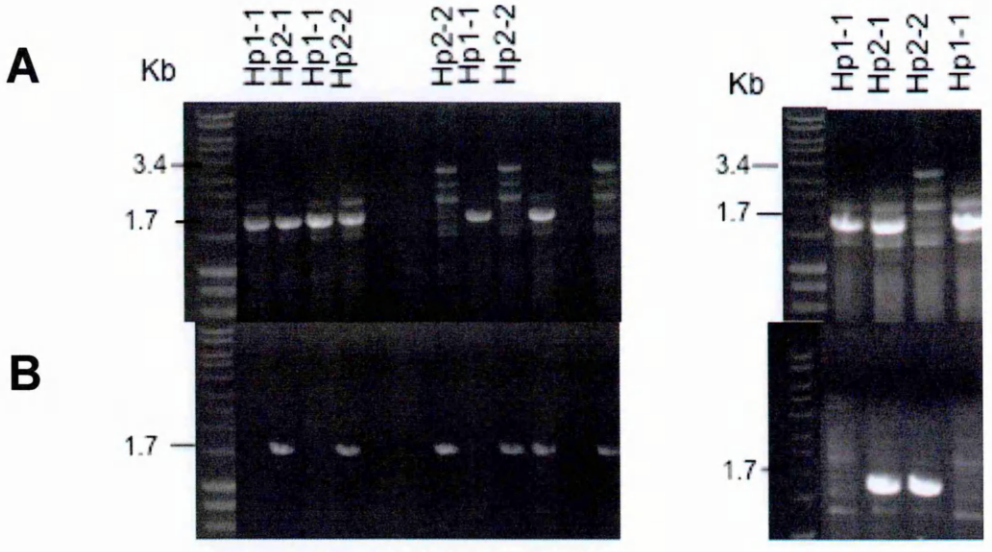
Results in this chapter were based on the clinical characteristics of the subjects in Table 5.1. In this experimental design, each disease group is further sub-grouped based on individual Hp phenotypes. For example, individuals with SMA are sub-grouped as Hp1-1, Hp2-1 and Hp2-2 SMA groups. But for the purpose of clarity and to avoid ambiguity, special nomenclatures such as SMA1-1, DC2-2 etc are used to describe the sub-groups and represent both the disease and Hp phenotype.

Table 5.1 Clinical characteristics of the subjects

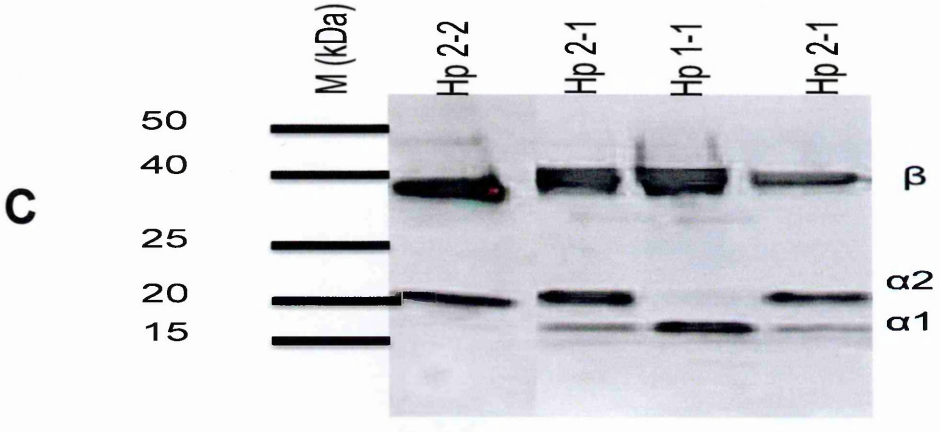
Groups	Hp Phenotypes (N)	Sex M/F (N)	Age [(Median, 25-75 IQR) Months]	Parasite Density Median (IQR)	PCV Median (IQR)	PD0	PD28
UM	1-1 (28)	19/9	42(13-77)	33065 (2244-51861)	31(27-34)*	28	28
	2-1 (30)	19/11	34(22-73)	15246(2326-65923)	32(27-36)*	30	30
	2-2 (13)	7/6	47(21-75)	21538(1073-77922)	32(27-36)*	13	13
SEVM	1-1 (12)	7/5	29(19-41)	42495(1271-86816)	20.50(18-27.25)*	12	12
	2-1 (16)	11/5	46(23-64)	38477(2339-114980)	25(20-31.75)*	16	16
	2-2 (9)	2/7	46(17-90)	1901(1084-55887)	27(21.50-320.50)*	9	9
CM	1-1 (17)	9/8	57(46-71)	16237(1852-93827)	28(25.50-34)*	17	17
	2-1 (19)	11/8	38(36-60)	23266(1109-82769)	25(22-28)*	19	19
	2-2 (4)	2/2	54(26.25-149.30)	1589(368-90790)	22(17.25-29)*	4	4
SMA	1-1 (18)	15/3	28(24-60)	10177(612-116567)	14(10.50-15)	18	18
	2-1 (12)	9/3	43(39-87.75)	39498(9139-89600)	12(10-14)	12	12
	2-2 (6)	4/2	39(29.50-4158.50)	46857(20912-142999)	15(11.75-16.50)	6	6
DC	1-1 (19)	10/9	62(40-143)	NA	26(22-30)*	19	19
	2-1 (29)	15/14	65(27-107)	NA	28(24-36)*	29	29
	2-2 (10)	4/6	83(23-117)	NA	28(16-37)*	10	10
CC	1-1 (38)	15/27	72(47-95)	NA	33.5 (30-35)*	38	38
	2-1 (61)	33/28	60(33-96)	NA	34(32-36)*	61	61
	2-2 (28)	13/15	72(4-106)	NA	37(32-39)*	28	28

N= Numbers; NA= Not Applicable; PCV= pack cell volume; IQR = Interquartile range; PD = Parasite density; * = statistically higher to PCV in the SMA. The Age, PD and PCV are shown as median and 25^{th}-75^{th} percentile. The PD across the entire Hp phenotypes is not statistically different. PCV in the SMA group are significantly lower to that of all other groups. All result presented in this chapter are based on this clinical characteristic.

Figure 5.1 Hp genotyping and Phenotyping. (A) The 3.4 and 1.7 kbs DNA band from primers Sa5 and Sa6; B) The 1.7kb specific DNA band for primers LS1 and LS2 specific for Hp2 allele and C) Western blot for Hp phenotypes



Primers Sa5 and Sa6 were designed to produce the 3.4 and 1.7kb specific band for both Hp1 and Hp2 (Fig.5.1A) but it hardly produce the 3.4 kb band, particularly in the Hp2-1 allele, hence, the Hp2 allele in both Hp2-1 and Hp2-2 was detected by a 1.7kb band from the LS1 and LS2 Hp2 specific primers (Fig. 5.1B). Both the product of Sa5/Sa6 and LS1 and LS2 were used to determine the Hp genotypes. An additional band in the Hp2-2 lane less than the 3.4kb could be due to random deletions in the second Hp2 allele.



Hp Phenotypes determined by western blot. M (kDa) = represent the molecular weight. The β, α2 and α1 represent the beta, alpha 2 and alpha 1 chains respectively. Western blot was performed to determine the Hp phenotypes in subjects with only plasma samples.

5.2 Genotyping and phenotyping for Hp

Both pairs of primers, Sa5/Sa6 and LS1/LS2 should produce distinct bands of the right size but Sa5 and Sa6 could not produce the 3.4kb band in the Hp2-1 (Fig. 5.1A) such that the presence of Hp2 in both Hp2-1 and Hp2-2 was detected by the 1.7kb band produced by LS1 and LS2 specific primers (Fig. 5.1B). Sa5 and Sa6 produced a 1.7kb Hp1 band present in both Hp1-1 and Hp2-1 genotypes and a 3.4kb Hp2 band present in Hp2-2 genotypes (Fig.5.1A)..The LS1 and LS2 primers took the advantage of the presence of an additional sequence/region on the Hp2 allele that is absent from the Hp1 allele (Fig. 2.1).

The western blot of Hp produces a stronger α2 band compared to α1 band in the presence of both α1 and α2 bands as in the Hp2-1 phenotype (Fig. 5.1C). However, the α1 band is as strong as the α2 band in the absence of α2 as in the Hp1-1 phenotype (Fig. 5.1C).

5.3 The frequencies of Hp phenotypes in malaria are not different to DC

The frequency of Hp1-1 is higher in the entire malaria and the DC groups compared to the CC group while that of Hp2-2 is lower in the malaria group than the CC except in the SEVM group (Table 5.1). The CM and SEVM groups have the highest frequencies of Hp1-1 and Hp2-2 respectively (Table 5.1). The trends of frequencies of Hp phenotypes in malaria groups are not different to those in the DC group (Table 5.1).

Hp2-1 was of the highest frequency in the entire population while Hp2-2 has the lowest frequency (Table 5.1). The observed frequencies in all the disease groups are not statistically different from the expected frequencies; therefore the phenotypes in the populations are at Hardy-Weinberg equilibrium (Table 5.1).

5.31: Hp phenotypes correlated differently and independent of the disease groups with PD, Hp, HPx and fHb.

Hp phenotypes correlated differently and independent of disease groups with pathological parameters such as PD, Hp, HPx and fHb. There is a significant negative correlation between PD and both fHb and Hp in the Hp2-1 phenotype (Table 5.2B). The same negative but not statistically significant correlations are typical of Hp1-1 phenotype (Table 5.2A). A negative and a positive but not significant correlation are observed between PD and both fHb and Hp respectively, in the Hp2-2 phenotype (Table 5.2C).

There is no correlation between PD and HPx level in the Hp1-1 phenotype (Table 5.2A), a significant negative correlation in Hp2-2 and a negative correlation in Hp2-1 (Table 5.2B-C). There are no significant correlations between fHb and Hp in the entire phenotypes (Table 5.2A-C). Hp positively and significantly correlates with HPx in the entire phenotypes (Table 5.2A-C). PD density significantly and negatively correlates with age only in the Hp2-2 phenotype (Table 5.2C).

Though the Hp2-1 phenotype has the highest PD, the PDs among Hp phenotypes are not significantly different (Table 5.2 footnote).

Table 5.2 Percentage frequencies of Hp phenotypes in malaria syndromes

Malaria groups	Observed Frequency And Number			Total	Expected Frequency and Number			P. Value at 2d.f
	Hp1-1 (N)	Hp2-1 (N)	Hp2-2 (N)		Hp1-1 (N)	Hp2-1 (N)	Hp2-2 (N)	
CC*	0.2992(38)	0.4803(61)	0.2205(28)	127	0.2909(37)	0.4969(63)	0.2122(27)	ns
UM*	0.3610(74)	0.4585 (94)	0.1805(37	205	0.3484(71)	0.4837 (99)	0.1679 (34)	ns
SEVM*	0.3500(21)$^{\$}$	0.4167(25)	0.2333(14)$^{\$}$	60	0.3118(19)	0.4932(30)	0.1951(12)	ns
CM*	0.4130(38)$^{\$}$	0.4674(43)	0.1196(11)	92	0.4182(38)	0.4570(42)	0.1248(11)	ns
SMA*	0.3482(39)	0.4821(54)	0.1696(19)	112	0.3472(39)	0.4840(54)	0.1686(19)	ns
DC*	0.3276(19)$^{\$}$	0.5000(29)$^{\$}$	0.1724(10)	58	0.3336(19)	0.4880(28)	0.1784(10)	ns

* The allele frequency and phenotypes in these populations are at Hardy-Weinberg Equilibrium, $= higher to CC; N= number; ns = not significant p-value computed from chi-square. The result presented here is from both genotyping by PCR and the phenotyping by western blot experiment. Due to the combination of both the genotype and the phenotype result, the number of subjects with Hp1-1, 2-1 and 2-2 as presented here are higher than that shown in table 5.1.

Table 5.3 Correlation between Hp phenotypes and PD, fHb, HPx, Hp and Age independent of disease group

Pathological Metrics	A. Hp1-1			B. Hp2-1			C. Hp2-2		
	r	95% c.i	p	r	95% c.i	p	r	95% c.i	p
PD and fHb	-0.010	-0.245_0.225	0.384	-0.149	-0.313_0.024	0.040*	-0.083	-0.363_ 0.212	0.286
PD and HP	-0.007	-0.305_0.159	0.256	-0.261	-0.448_-0.054	0.005**	0.064	-0.223_0.379	0.327
PD and HPx	0.085	-0.119_0.282	0.201	-0.175	-0.373_0.039	0.040*	0.242	-0.051_0.505	0.040*
fHb and HP	-0.136	-0.334_0.100	0.093	0.061	-0.113_0.230	0.242	-0.029	-0.315_0.262	0.422
HP and HPx	0.509	0.342_0.644	<0.0001****	0.530	0.394_0.644	<0.0001****	0.451	0.193_0.651	0.0004***
Age and PD	0.085	-0.151_0.312	0.341	-0.021	-0.229_0.190	0.422	-0.355	-0.612_-0.0303	0.014*

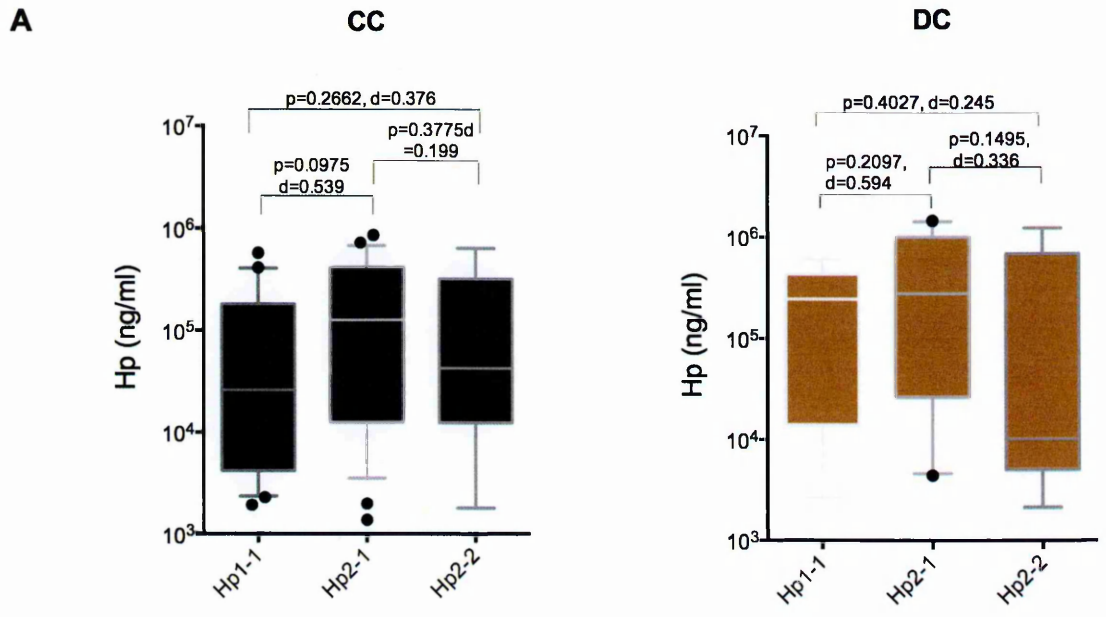
_(Underscore) indicate range. * =Statistically significant. c.i= Confidence interval.
The parasite densities (PD) are not statistically different across the phenotypes. The median (IQR; 10-90 percentile) PD values are; Hp2-2= 33505(2438-73094), Hp2-1=44800(2239-82771) and Hp1-1= 39890 (3368-74720).

5.4 There are low and stable levels of plasma Hp in both SMA1-1 and SMA2-1 groups

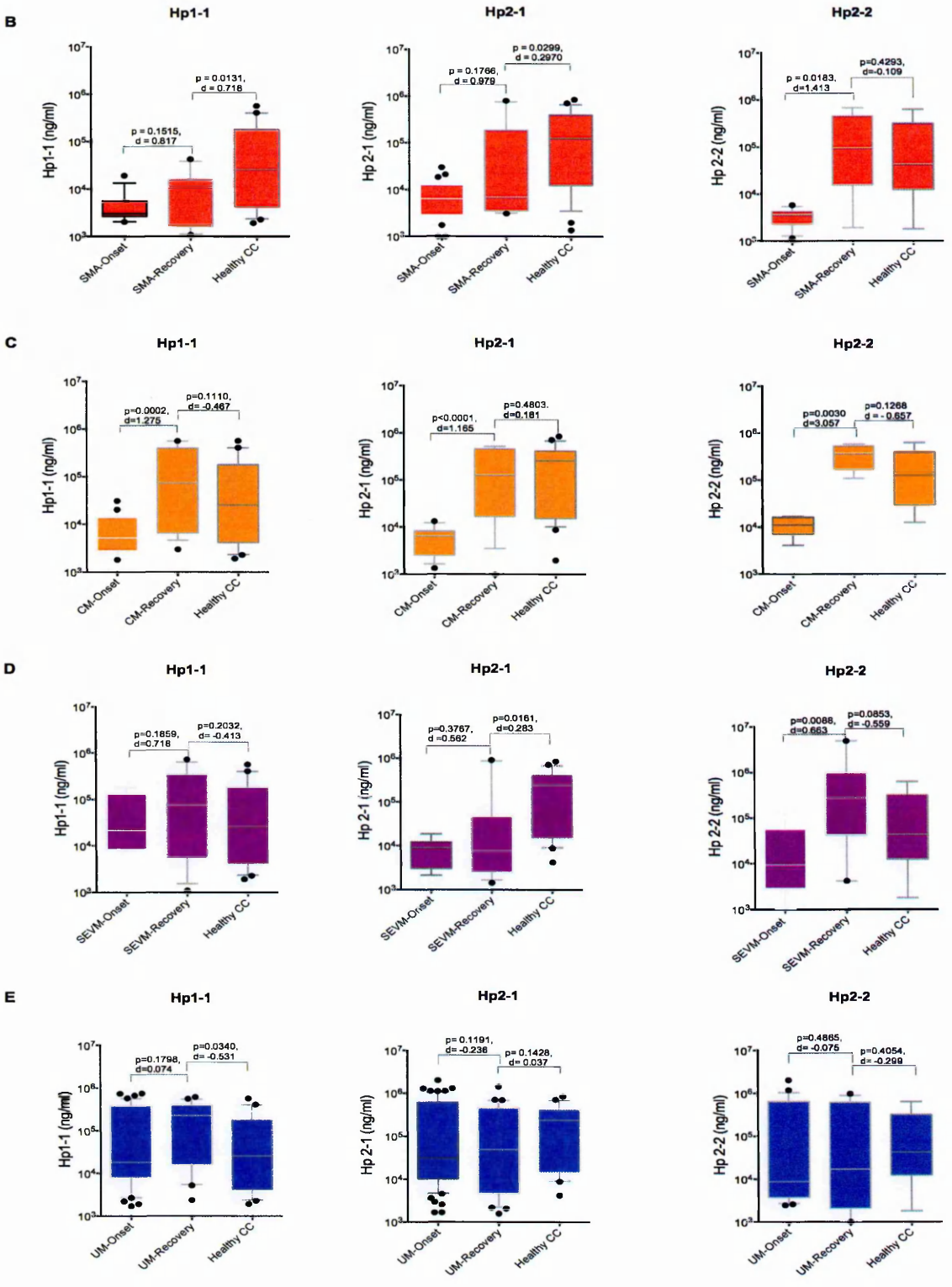
The levels of Hp in the entire Hp phenotypes are not statistically different within the control groups. However, Hp level in the DC2-2 group has a lower median compared to levels in both DC2-1 and DC1-1 groups (Fig. 5.2A).

Plasma Hp level at acute onset of the disease is lower in the SMA2-2 group than level in the SMA2-1 and the SMA1-1 unlike with the phenotypes in the CM groups at acute onset (Fig. 5.2B-C). While the Hp level at recovery in the SMA2-2 group is not different to level in the CC2-2 group, the levels in both SMA1-1 and SMA2-1 remain lower even after the full recovery of patients when compared to levels in the healthy CC1-1 and 2-1 groups (Fig. 5.2B). Plasma Hp levels at recovery in the SMA 1-1, SMA 2-1 and SEVM2-1 groups are significantly lower to those of the CC1-1 and CC2-1 groups (Fig. 5.2B and D).

Figure 5.2 Levels of Hp phenotypes in the A) controls; CC and DC; B) SMA; C) CM; D) SEVM and E) UM



Graphs are presented as median interquartile range with 10-90 percentile. Outliers beyond 10-90 percentile are indicated as round black dots on the graph. Non-parametric un-paired t-test was used to compare two groups while Non-parametric ANOVA was used to make comparison between the three groups.



The black = SMA; Brown = DC; Red = SMA; Orange = CM; Purple = SEVM and Blue = UM. Figures 5.2 B-E are presented as median interquartile range with 10-90 percentile. Points beyond 10-90 percentile are indicated as round black dots on the graphs. An unpaired nonparametric Mann-Whitney test was used to compare data between two non-paired groups such as data at recovery and the CC. A paired nonparametric Wilcoxon test was used for paired data within each disease group such as paired PD0 and PD28

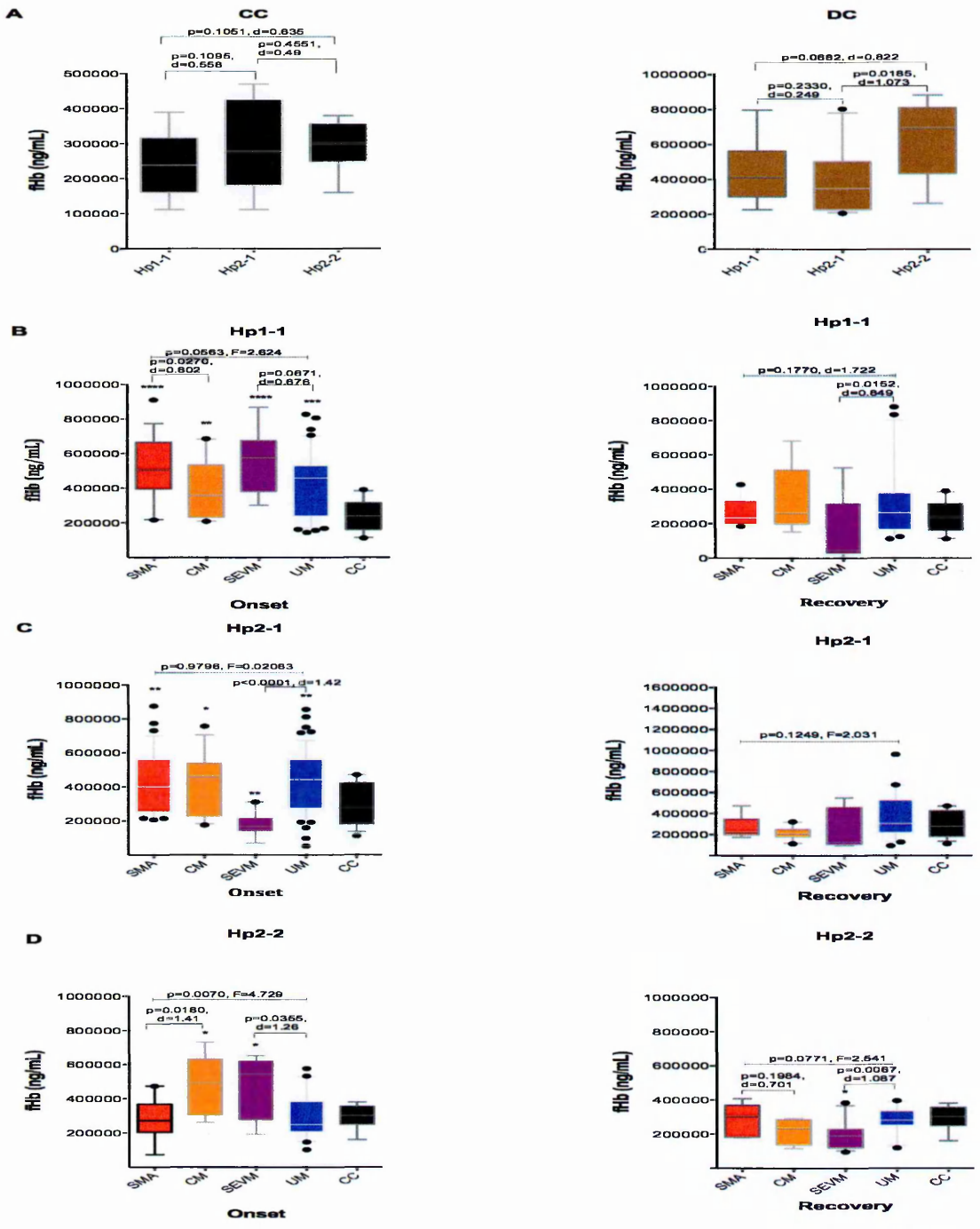
These data are based on the numbers of subjects in table 5.1.

5.5 SMA2-2 and UM2-2 have low plasma fHb level at acute onset

Both SMA2-2 and UM2-2 groups have lower fHb levels at onset compared to CM2-2, SEVM2-2 (Fig. 5.3D), SMA1-1 and UM1-1 (Fig. 5.3B-D). The plasma fHb levels in both SMA2-2 and UM2-2 groups are essentially the same both at onset and recovery and are not different to CC2-2 group (Fig. 5.3D).

Within the control groups, the fHb levels in the entire phenotypes are not statistically different, however, the level in both the CC2-2 and the DC2-2 groups show higher median; the level in the DC2-2 is statistically higher to both DC1-1 and DC2-1 (Fig. 5.3A). The higher fHb level in the DC2-2 is accompanied by lower Hp level (Fig. 5.3A and 5.2A). Both fHb and Hp levels are stable in the UM2-2 group (Fig. 5.2E and 5.3D). In the SMA2-2 group the levels of fHb at onset and recovery are the same, however its Hp level is low at onset but high on recovery (Fig. 5.2B and 5.3D) and appears not to be associated with fHb scavenging at onset. The plasma fHb levels in both the CM2-2 and SEVM2-2 are higher at onset but lower at recovery compared to the levels in the SMA2-2, UM2-2 and CC2-2 groups (Fig. 5.3D).

Figure 5.3 Levels of fHb associated with each Hp phenotype group in the A) controls, CC and DC; and levels associated with the malaria syndromes in subjects with B) Hp1-1; C) Hp2-1 and D) Hp2-2



= Statistically different to CC (=P<0.05; **=P<0.01; ***=P<0.001; ****=P<0.0001). Data are presented as median interquartile range with 10-90 percentile. Points beyond 10-90 percentile are indicated as round black dots on the graphs. Non-parametric Mann Whitney test was used to compare between two groups while ANOVA with multiple comparison testing was used to compare more than two groups. Pvalue <0.05 was considered as statistically significant. F=effect size and do indicate differences in mean values. The numbers of subjects in each group are as indicated in table 5.1.

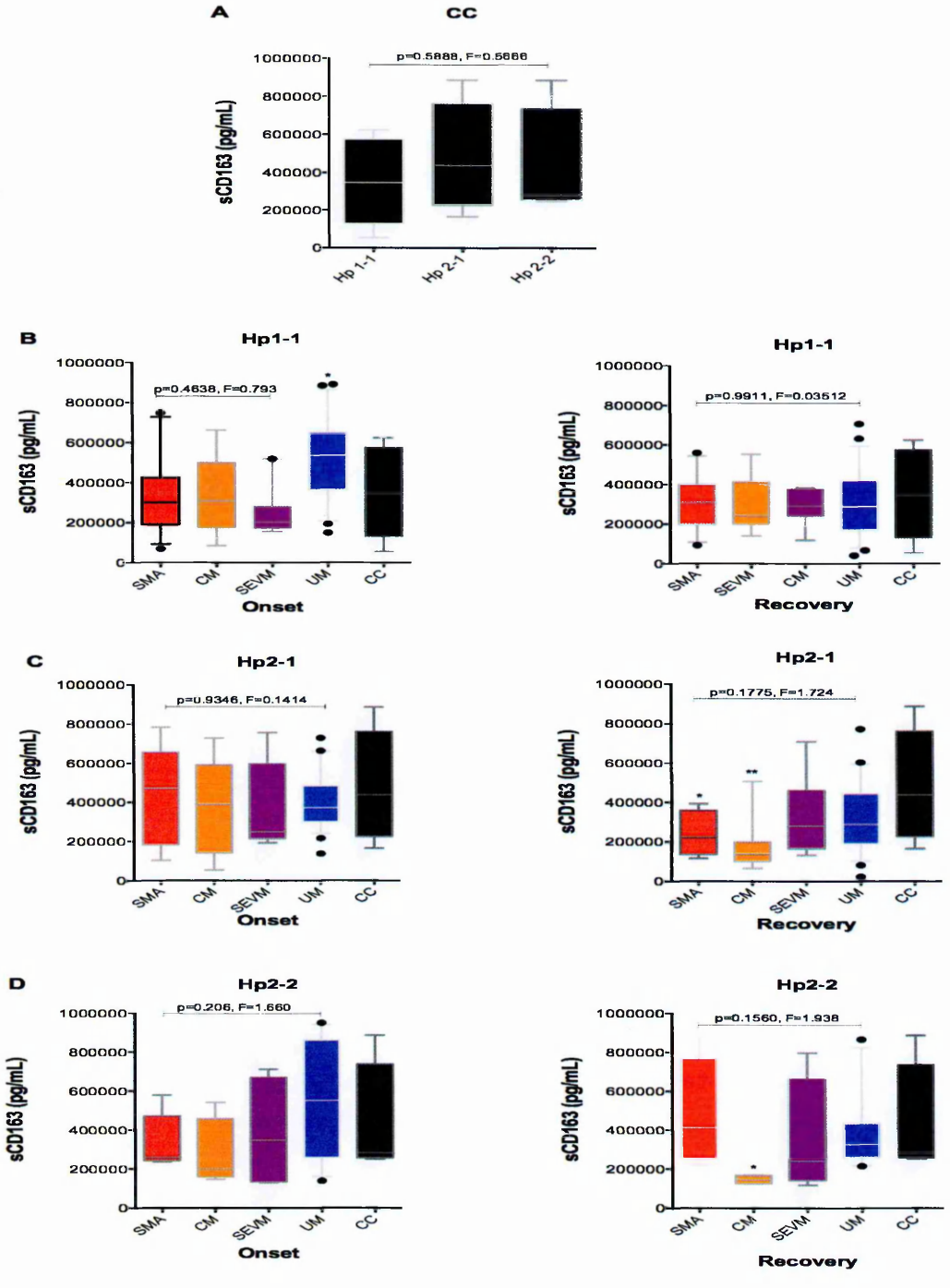
5.6 Severe malaria groups with Hp1-1 have stable sCD163 levels

The levels of sCD163 are not statistically different in the entire Hp phenotypes within the CC group (Fig. 5.4A). However, Hp1-1 individuals within the severe malaria groups have stable levels of sCD163 that are not different to levels in the CC1-1 group at both onset and recovery (Fig. 5.4B). On the other hand, the level of sCD163 in the UM1-1 group at onset is significantly higher than the level in the healthy CC1-1 group and those within the severe malaria groups (Fig. 5.4B).

The levels of sCD163 at onset are not statistically different in the entire malaria groups with both Hp2-1 and Hp2-2 phenotypes (Fig.5.4C-D). However, malaria recovered patients with Hp2-1 phenotype, particularly the SMA2-1 and CM2-1 groups have lower levels of sCD163 in their healthy state compared to the levels in the disease state and the CC2-1 group (Fig 5.4C).

While the sCD163 level in the CM2-2 group is lower at recovery to the level in the CC2-2 group (Fig. 5.4D), the level in the SMA2-2 group is higher at recovery to the levels at onset and the CC2-2 groups (Fig. 5.4D).

Figure 5.4 Levels of sCD163 associated with each Hp phenotype. A) Levels in the control; and levels associated with B) Hp1-1; C) Hp2-1; D) Hp2-2



= Statistically different to CC (=p<0.05; **=p<0.01). Data are presented as as median interquartile range with 10-90 percentile. Points beyond 10-90 percentile are indicated as round black dots on the graphs. Non-parametric Mann Whitney test was used to compare between two groups while ANOVA with multiple comparison testing was used to compare more than two groups. p-value <0.05 was considered as statistically significant. F=effect size and do indicate differences in mean values. The numbers of subjects in each group are as indicated in table 5.1.

5.7 Plasma Hpx levels recovered in the entire malaria and Hp phenotypes unlike plasma Hp levels.

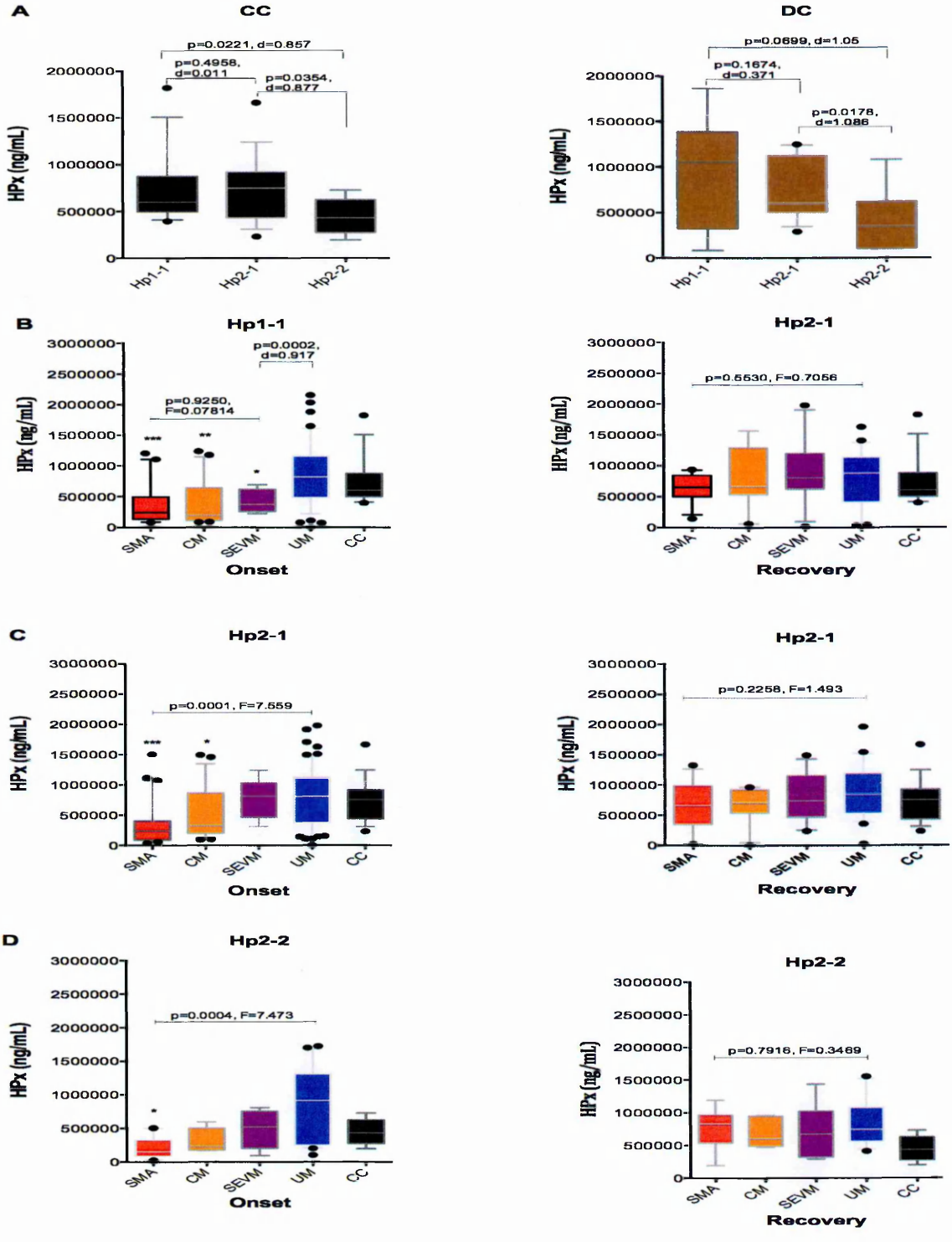
In the two control groups, the level of HPx associated with Hp2 allele 6:is not statistically different to the CC2-1 group, the level in the CC2-2 group is significantly lower than the levels in both CC1-1 and CC2-1 groups (Fig. 5.5A). On the other hand, plasma HPx level in the DC2-1 group is significantly lower than the DC1-1 group while the level in the DC2-2 group is lower than the DC2-1 group (Fig 5.5A).

Unlike in the control groups, the plasma HPx levels in individuals with Hp1-1 phenotype and presenting with severe forms of malaria (SMA1-1, CM1-1 and SEVM1-1) are all the same at acute onset and are significantly lower than the levels in the UM1-1 and the healthy CC1-1 groups (Fig.5.5B). However, the levels at recovery are not statistically different in the entire disease groups (Fig. 5.5B). HPx levels are lower at acute onset in both SMA2-1 and CM2-1 groups compared to the level in the healthy CC2-1 group (Fig. 5.5C), however the levels are not statistically different at recovery (Fig. 5.5C).

The plasma HPx levels in both the SMA2-2 and CM2-2 groups are statistically lower at onset compared to the level in the CC2-2 group (Fig. 5.5D). However, HPx levels in the entire Hp2-2 phenotype with malaria are the same at recovery and are higher than the levels in the CC2-2 group (Fig. 5.5D).

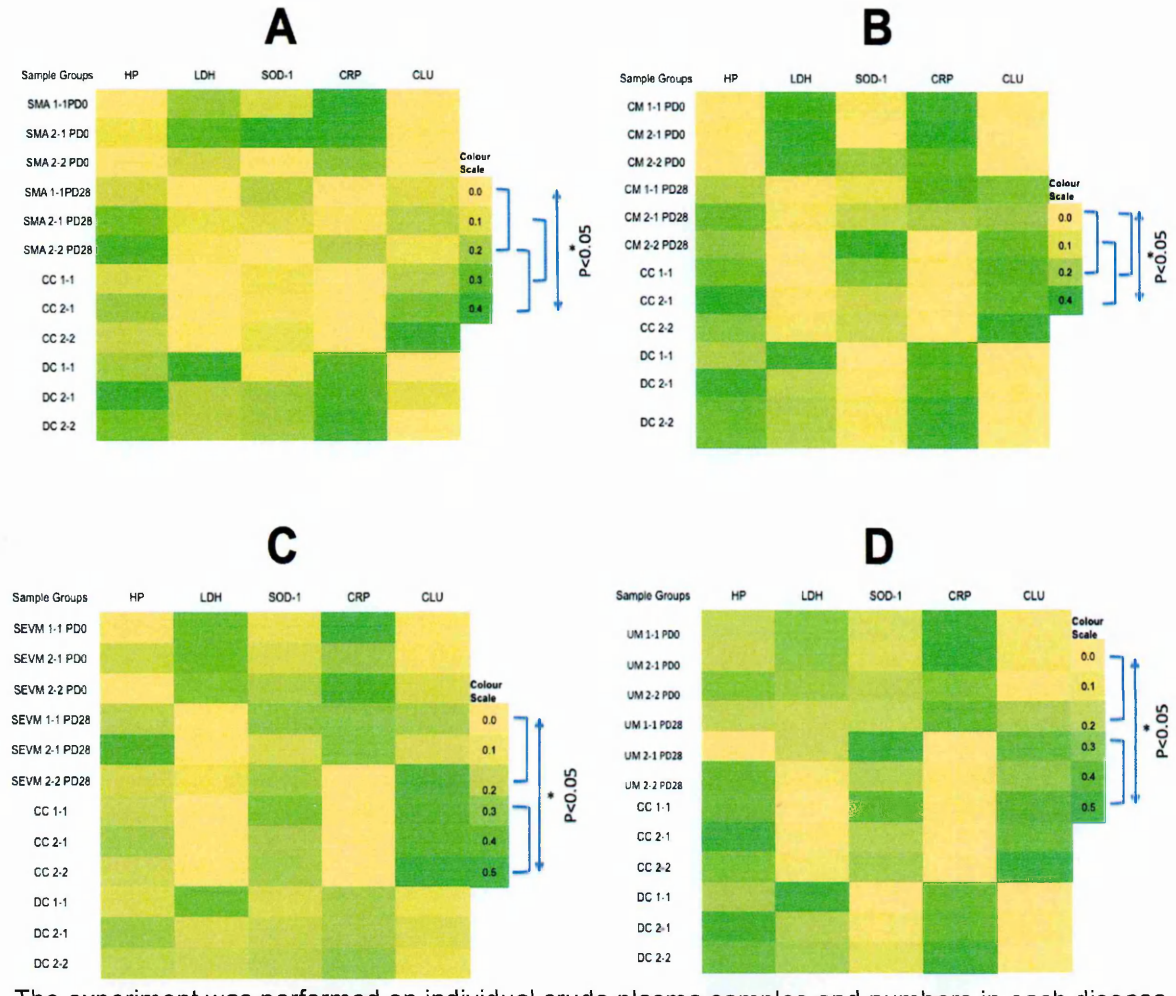
While the plasma HPx levels, particularly in both the Hp2-1 and Hp2-2 phenotypes show distinct differences and appear to be discriminatory among the malaria groups at acute onset (Fig. 5.5A-D), levels all returned to the same point on recovery of the patients irrespective of the differences in both the Hp and malaria phenotypes (Fig.5.5B-D).

Figure 5.5 Levels of HPx in each Hp phenotypes A) in the controls; B) Hp1-1; C) Hp2-1 and D) Hp2-2



= Statistically different to CC (=P<0.05; **=P<0.01; ***=P<0.001). Data are presented as median interquartile range with10-90 percentile. Points beyond 10-90 percentile are indicated as round black dots on the graph. Non-parametric Mann Whitney test was used to compare between two groups while non-parametric ANOVA was used to compare more than two groups. p-value <0.05 was considered as statistically significant. The numbers of subjects in each group are as indicated in table 5.1

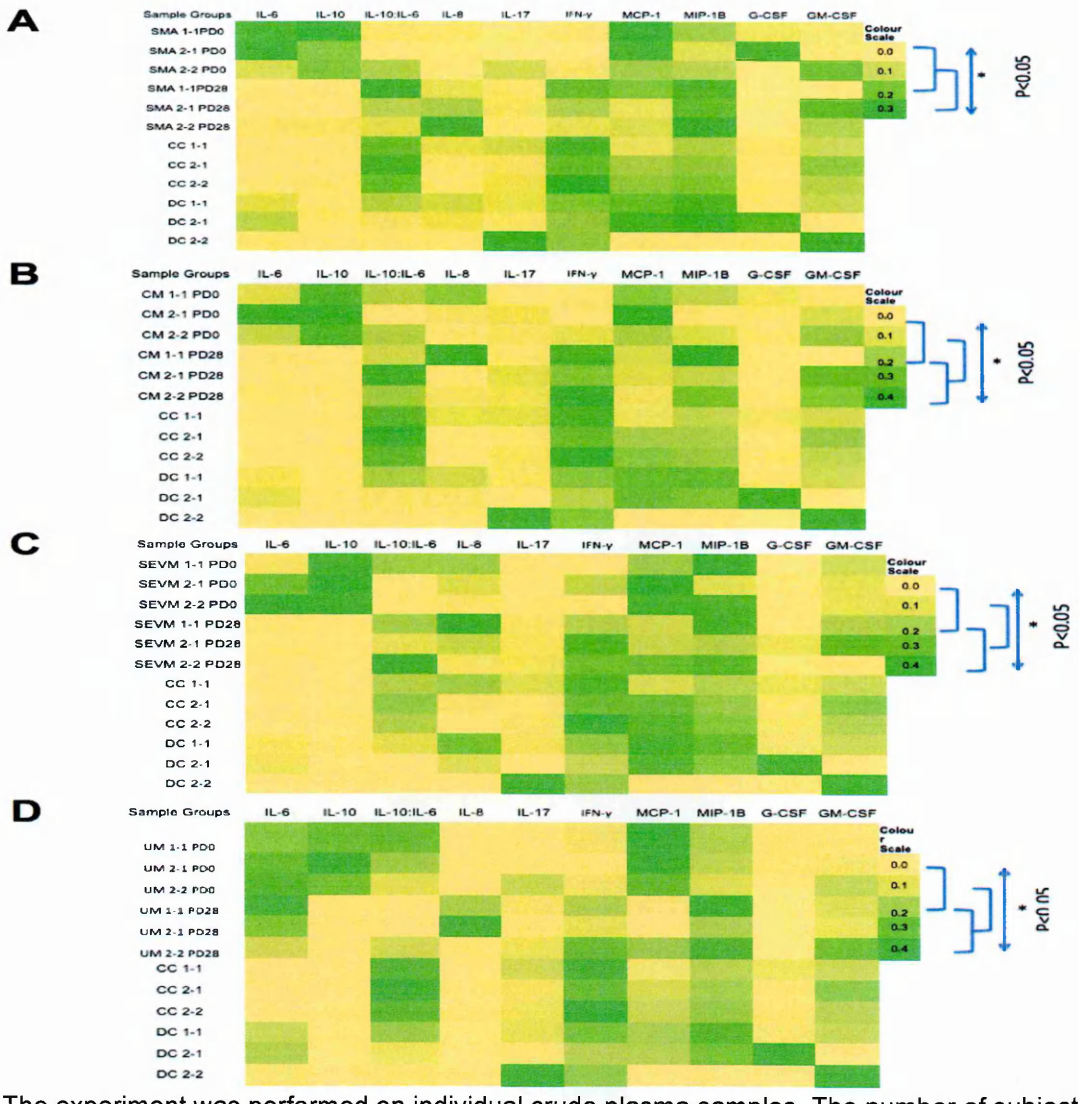
Figure 5.6 Plasma levels of markers of oxidative stress and inflammation associated with Hp phenotypes in A) SMA; B) CM; C) SEVM and D) UM groups



The experiment was performed on individual crude plasma samples and numbers in each disease groups are as shown in table 5.1. The data were normalized by dividing each data point by the column total and the values obtained were then summed up by row and each data point is again divided by the row total. The control groups were normalized alongside with each disease groups to avoid bias and ensure comparison within each group. Hence, slight but not significant variations in levels across the control groups exist when comparing between groups. This is because values used for normalization within each group are different. After normalization, we analyzed the data by multiple comparison testing. The colour scale shows the intensity or level of expression within each protein group on the heatmap. The levels of significance are as indicated on the colour scale, which reflect the colour intensity that are significantly different within each group.
A $p<0.05$ was considered as statistically significant.
HP=Haptoglobin; LDH=Lactate dehydrogenase; SOD-1=Superoxide dismutase; CRP=C-reactive protein; CLU=clusterin.

Figure 5.7 Plasma levels of cytokines associated with Hp phenotypes in the

A) SMA; B) CM; C) SEVM; D) UM



The experiment was performed on individual crude plasma samples. The number of subjects in each disease group is as shown in table 5.1. Normalization was performed by dividing each data point by the column total and the values obtained were then summed up by row and each data point is again divided by the row total. The control groups were normalized alongside with each disease groups to avoid bias and ensure comparison within each group. Hence, slight but not significant variations in levels across the control groups exist when comparing between groups because values used for normalization within each group are different. The colour scale shows the intensity or level of expression within each protein group on the heatmap. After normalization, we analyzed the data by multiple comparison testing. The levels of significance are as indicated on the colour scale, which reflect the colour intensity that are significantly different within each group. A $p<0.05$ was considered as statistically. IL= Interleukin; IFN-γ = Interfron gamma; MCP-1=Monocyte chemoattractant protein-1; MIP-1β= Macrophage Inflammatory protein-1 (chemokine c-c motif ligand 4; CCL4); G-CSF=Granulocyte-colony stimulating factor; GM-CSF=Granulocyte macrophage colony stimulating factor

Table 5.4 Summary of observation from Figs. 5.6 and 5.7

Hp phenotypes and Disease Groups		LDH	CRP	SOD-1	CLU	IL-10/IL-6	IL-17 and IFN-Y	Macrophage associated (MCP-1, MIP-1B and GM-CSF) and G-CSF
SMA	Hp1-1	Level at PD0 higher to levels in the CCs and at PD28	High level at PD0. Levels at PD28 not different to CCs	Higher level at PD0 relative to PD28 and CCs	Level at PD0 lower than CC but not different to PD28 in SMA 2-2	Level s at PD0 depleted relative to PD28 and CCs	IL-17 are low in entire group at PD0 and are not different to levels at both PD28 and CCs. However, levels at PD0 in HP2 allele in the SMA, CM and UM are slightly higher compared to Hp1. IFN-Y level is generally low at PD0 but levels at PD28 are not significantly different to CCs	MCP-1 is higher in Hp1 allele at PD0 relative to CCs. G-CSF is uniquely and significantly higher in SMA2-1 at PD0 compared to level at both PD28 and CC2-1. G-CSF levels is also high in DC2-1
	Hp2-1							
	Hp2-2		Lower levels at PD0 compared to Hp1 allele but levels at PD0 not different to PD28 and higher to CC	No SOD-1 at both PD0 and PD28		Level at PD0 not different to PD28 and CC2-2		GM-CSF is higher at PD0 compared to PD28 and CC2-2. Higher level is also found in DC2-2
CM	Hp1-1	Level at PD0 is higher compared to CCs, PD28 other groups.	Level in Hp1-1 at PD0 is not different to PD28. Levels in both Hp1-1 and 2-1 at PD28 are significantly higher to CCs	Slightly depleted at PD0 compared to CCs	Low level at PD0. Levels Significantly upregulated from PD0 to PD28. Level at PD28 not different to CCs	Levels at PD0 not very different to CC1-1		G-CSF levels across the entire phenotypes in CM, SEVM and UM groups at PD0 are not different to level at PD28 and CCs. MCP-1 level is significantly higher in CM2-1 group at PD0 compare to levels at PD28 and CC2-1. MCP-1 is significantly higher in the entire Hp phenotype at PD0 in the UM compared to levels at both PD28 and CCs. G-CSF level in the CM, SEVM and UM at PD0 are not different to levels at PD28 and CCs
	Hp2-1					Level at PD0 depleted relative to CC2-1		
	Hp2-2		Levels at PD28 not different to CC2-2	Higher level at both PD0 and PD28 compared to CC2-2		Level at PD0 not very different to CC2-2		
SEVM	Hp1-1	Levels at PD0 are higher to CCs and PD28	Levels at PD28 not different to PD0 in Hp2-1. Levels in both Hp1-1 and 2-1 are significantly higher to CCs	Levels at PD0 are not different to PD28 and CC	Levels at PD0 are lower compared to Ccs	Level at PD0 not different to CC1-1		
	Hp2-1					Levels at PD0 depleted in Hp2 allele relative to control		
	Hp2-2		Levels at PD28 not different to CC					
UM	Hp1-1	Levels in the entire phenotypes are higher to CCs	Levels at PD 28 not different to PD0 and higher than CC	Levels at PD0 is not statistically different to levels at PD28 and CC	Level at PD0 slightly lower than PD28 and CCs	Higher levels at PD0 are higher compared to PD28 particularly in Hp1 allele but levels at PD0 not different to CCs		
	Hp2-1		Levels at PD28 not different to CC	Higher at PD28 compared to CC2-1				
	Hp2-2			Levels at PD0 is not statistically different to levels at PD28 and CC				

This table summarizes the observations in fig. 5.6 and 5.7. Cells are merged for observation(s) common to more than one Hp phenotypes or Hp allele within or across the disease groups. CCs = controls; IL= Interleukin; IFN-γ = Interfron gamma; MCP-1=Monocyte chemoattractant protein-1; MIP-1β= Macrophage Inflammatory protein-1 (chemokine c-c motif ligand 4; CCL4); G-CSF=Granulocyte-colony stimulating factor; GM-CSF=Granulocyte macrophage colony stimulating factor

5.80 Plasma levels of the markers of oxidative stress, inflammation and cytokines are dependent on both malaria and Hp phenotypes

The plasma level of SOD-1 differs with both Hp and malaria phenotypes (Fig. 5.6A-D). There is null SOD-1 level in the SMA2-2 group at acute onset and high level of IL-10:IL-6. Aside the null SOD-1 level in the SMA2-2 group at acute onset, the rest of the malaria groups with Hp2-2 phenotype have either higher level of SOD-1 at acute onset or levels that are not different to that at both PD28 and the CC as well as null or stable levels of IL-10:IL-6 (Fig. 5.6A-D; Table 5.4).

On the other hand the higher levels of SOD-1 in the SMA1-1 and 2-1 are accompanied by null IL-10:IL-6 ratio (Fig. 5.6A and 5.7A; Table 5.4), while the stable to depleted level of SOD-1 in subjects with Hp1 allele and presenting with CM, SEVM and UM are associated with stable and high IL-10:IL-6 at acute onset; PD0 (Fig. 5.7A-D; Table 5.4).

Furthermore, the level of CRP in the SMA2-2 at PD28 is higher than CC unlike the rest of malaria groups with Hp2-2 while the CRP levels in SMA1-1 and 2-1 at PD28 are not different to CC but level in the rest of malaria groups with Hp1-1 and/or 2-1 have higher levels of CRP at PD28 compared to the CC (Fig. 5.6A-D and Table 5.4).

Both the levels of Hp and Clusterin (CLU) are largely depleted in the entire severe malaria groups at acute onset (Fig. 5.6A-C).

There are higher levels of LDH in the CM groups at acute onset compared to other malaria groups, except the SEVM2-1 group (Fig. 5.6C; Table 5.4).

The levels of CRP are slightly lower in the Hp2-2 phenotype with malaria, except in the SEVM2-2 group (Fig. 5.6A-D).

While the level of LDH is higher in the DC1-1, its SOD-1 level is lower compared to the DC2-1 and the DC2-2 (Fig. 5.6D). There are higher levels of both

CLU and Hp in the DC2-1 group compared to the DC1-1 and DC2-2 groups (Fig. 5.6A-D).

The levels of IL-8 are depleted at acute onset (PD0) in the entire CC, UM and SMA groups independent of Hp phenotypes. However, at recovery, both SMA2-2 and UM2-1 groups have high plasma levels of IL-8 compared to levels in the CCs (Fig. 5.7A and D).

Levels of IL-17 is slightly higher in SMA2-2, DC2-2 and subjects with Hp2 allele in the CM and UM groups compared to levels in Hp1 allele and controls (Fig. 5.7A, B and D).

Higher levels of GM-CSF compared to that of the control are found in SMA2-2 and CM2-2 (Fig. 5.7A-B). Levels of macrophage-associated chemokines such as MCP-1 and MIP-1β are as described in the summary table 5.4.

5.9 Discussion

Hp phenotypic frequencies in malaria as observed in our data have been previously reported (Atkinson et al., 2007, Elagib et al., 1998, Minang et al., 2004, Quaye et al., 2000b), but without satisfactory explanation for the observed association and whether the observation could be found with other disease. The claimed susceptibility of Hp1-1 to malaria is difficult to comprehend as it's expected to be protective (selected) against malaria due to its high frequency in the malaria holoendemic sub-Saharan Africa (Quaye et al., 2006, Teye et al., 2006, Elagib et al., 1998). While the high and low Hp phenotypic frequencies appear to be associated with susceptibility and protection in malaria syndromes, the same frequencies are present in the DC group. This implies that the associations are not only restricted to malaria infection neither are they a consequence of the infection. More so, that the populations were at Hardy-Weinberg equilibrium. We propose that though malaria is one of the major selective pressures and evolutionary driving forces (Kwiatkowski, 2005, Cox et al., 2007), Hp phenotypes could be selective for something other than malaria despite its role in malaria pathophysiology.

The role of Hp in malaria could be more associated with the levels of the protein than the frequencies of the phenotypes. The phenotypes could be capable of modulating response to infection. The result indicated that the susceptibility of Hp1-1 to SMA is not unconnected with the generally low and stable plasma Hp level associated with the phenotype (Fig. 5.2B). Levels of PD and Hp measured independent of malaria disease groups indicated that Hp levels could either decrease or increase together with PD in the subjects with Hp1 allele but not in the Hp2-2 group (Table 5.3) and these differences could be important in the pathological processes.

The Hp2-2 phenotype could be more protective against the severe forms of malaria than the Hp1-1 phenotype. The negative correlation between age and PD in the Hp2-2 phenotype (Table 5.3) could indicate decrease in parasite load with increase in age and vice versa. It could also be due to the anti-parasitic activity of Hp2-2 (Imrie et al., 2004). Previous reports have also showed that the Hp2-2 sera agglutinate *Streptococcus Pyrogenes* carrying a T4 antigen at higher agglutination titre compared to the Hp2-1 sera, while the Hp1-1 sera exhibited anti-agglutination function (Prokop et al., 1979, Gunther et al., 1979).

Hp phenotypes modulate response to malaria syndromes and appear to be an underlying factor for the differences in malaria pathophysiology among individuals presenting with the same or different malaria syndromes. Plasma Hp levels as well as levels of other plasma proteins of importance in malaria pathophysiology such as the marker of oxidative stress; intravascular haemolysis; chemokines and cytokines appear to be dependent on Hp phenotypes. As a result, individuals with different Hp phenotypes and the same malaria syndrome could mount different responses to the infection.

It is not clear why the levels of Hp and HPx are low in the SMA2-2 (Fig. 5.2D and 5.5D) in the absence of high fHb levels in the group (Fig. 5.3D). These low Hp and HPx levels in the SMA group with Hp2-2 (SMA2-2) are unlikely to be congenital as both the levels of Hp and HPx in the group recovered to normal at PD28 (Fig. 5.2D and 5.5D). It's likely that both The HPx and Hp levels in the SMA subjects with Hp2-2 (SMA 2-2 group) are involved in some other functional activity, which is likely to be responsible for their low levels in the face of low fHb. The low levels of plasma Hp at PD0 and PD28 in both SMA2-1 and SMA1-1 groups are likely to be congenital and account for the low Hp level through convalescence to full recovery in the SMA group as observed in the previous chapter (Fig. 4.1A and 5.2B). These observed congenitally low Hp levels are likely caused by certain Hp

variants known to be associated with high Hp levels but are of lower frequency in the SMA groups compared to the CC as further discussed in Chapter 6. In the event of a congenitally low level of Hp the level of plasma HPx is unaffected (Delanghe et al., 1998b), but this is only possible in the absence of underlying haemolysis. Therefore, the normal levels of HPx in the face of low Hp levels in both SMA1-1 and SMA2-1 groups at recovery are rather indicative of congenitally low plasma Hp levels.

The high plasma levels of both fHb and LDH in the CM2-1 and CM2-2 groups reflect higher IVH and nitric oxide scavenging than the SMA group. The CM group is characterized with mild anaemia coupled with impaired consciousness or coma (Mishra and Newton, 2009, Janka et al., 2010), characteristics that are directly or indirectly connected with elevated level of LDH. High LDH plasma levels have been linked to low NO and organ vasculopathy via impairment of blood flow (Kato et al., 2006, Gladwin et al., 2003, Reiter et al., 2002, Minneci et al., 2005), a proposed mechanism of CM (Idro et al., 2010). LDH is a biomarker of intravascular haemolysis (Kato et al., 2006, Ballas, 2015, Ballas, 2013, Tabbara, 1992) as well as Nitric Oxide (NO) bioavailability (Kato et al., 2006).

Though sCD163 is a marker of macrophage activation (Burdo et al., 2011b, Burdo et al., 2011a, Stilund et al., 2014, Chua et al., 2013b) with anti-inflammatory function (Adly et al., 2015, Frings et al., 2002), the stable levels of sCD163 from onset to recovery in the SMA1-1 group (Fig. 5.4B) are likely to be linked to null/low Hp level in the group and failure in the homeostatic scavenger-receptor pathways rather than impaired macrophage activation (Fig. 5.3B). Impaired macrophage in the SMA1-1 group is highly unlikely as a result of high level of MIP-1β (CCL4) in the group at both onset and recovery. On the other hand, the depleted levels of

sCD163 at recovery in the CM2-2 and CM2-1 groups (Fig. 5.4C-D) could indicate a yet unknown additional function of sCD163 during the recovery phase.

The null level of SOD-1 is indicative of absence of oxidative stress (Liu et al., 2016). Oxidative stress as indicated by high SOD-1 level and cytokine imbalance as indicated by low/stable IL-10:IL-6 appears to be the major underlying pathophysiology associated with malaria subjects with Hp2-2 except the SMA2-2 group, which is without oxidative stress and cytokine imbalance. However, inflammation in the SMA2-2 subjects appears to be poorly managed. On the other hand, cytokine imbalance and oxidative stress appears to be the underlying pathophysiology in the SMA1-1 groups unlike in other malaria subjects with Hp1-1. However, while inflammation was quickly managed in the SMA1-1 and 2-1 groups, it appears to persist in all other non-SMA malaria subjects with Hp1-1 and/or Hp2-1 phenotypes. We propose that cytokine balance and absence of oxidative stress proffers a better prognosis in SMA2-2 and non-SMA groups with Hp1 allele while persistent inflammation proffers a worse prognosis in these groups. Therefore, inflammation, cytokine imbalance and oxidative stress that are proposed as underlying factors for onset of severe malaria (Helleberg et al., 2005, Perkins et al., 2011) or progression from mild to severe forms of malaria (Burte et al., 2013, Kurtzhals et al., 1998) are not typical in all individuals but are rather dependent on Hp phenotypes.

The duration from the onset of the disease to recovery appears to be associated with Hp phenotypes; inflammation as indicated by CRP levels appear to have been effectively resolved at PD28 in the SMA1-1, 2-1 and Hp2-2 subjects in the non-SMA groups but not in the SMA2-2 and Hp1-1 subjects in the non-SMA groups (Fig. 5.6A-D, Table 5.4). While the 28th day post treatment might be sufficient for recovery of the patients based on clinical parameters such as PCV and symptoms resolution, there might still be some degree of ongoing

inflammatory response in the recovery phase in some subjects. This is evident by the higher level of CRP in both the SMA2-2 and Hp1-1 non-SMA subjects (Fig. 5.6A-D, Table 5.4).

Hp phenotype appears to explain the disparity in the levels of IL-10 reported in the SMA (Lyke et al., 2004, Burte et al., 2013). IL-6 is reported to act as an anti-inflammatory cytokine alongside IL-10 (Burte et al., 2013) but it also functions as a pro-inflammatory cytokine (Scheller et al., 2011, Prakash et al., 2006).

IL-8, which is produced by epithelial cells, muscle cells and endothelial cells (Hedges et al., 2000, Wolff et al., 1998, Utgaard et al., 1998) is likely to play some role during sporozoites invasion through the cells as with other invading parasites (Eickhoff et al., 2003, Li et al., 1998). The different pattern of IL-8 expression in the malaria groups at onset and recovery indicates a likely role in malaria; IL-8 might have a similar biologic function in both UM and SMA groups and another similar function in both CM and SEVM groups at onset (Fig. 5.7 A-D). Early IL-8 response is thought to confer protection but late production could be linked to disease severity (Lyke et al., 2004, Barnwell, 2006). However, this also appears to be Hp phenotype dependent.

Both IL-17 and IFN-γ have a synergistic role in inflammatory disease (Savarin et al., 2012, Teunissen et al., 1998), therefore, the depleted level of IL-17 and IFN-γ might constitute a worse prognosis. IL-17 might protects from SMA through its involvement in granulopoiesis, infiltration of neutrophils and recruitment of T-cell into peripheral tissue (Weaver et al., 2007). However, high IL-17 in the CM might be a disadvantage. Neutrophil infiltration enhances the sequestration of monocytes in the brain and could lead to cytokine imbalance (Che et al., 2000).

Plasma levels of MCP-1 are indeed independent of the anemic status as previously reported (Fernandes et al., 2008, Wilson et al., 2010) but are rather dependent on Hp and malaria phenotypes. The levels of both MCP-1 and IL-10

are high at acute onset and could be as a result of the migration and infiltration of IL-10 secreting monocytes to the site of active inflammation through MCP-1 signal (Deshmane et al., 2009).

The circulatory levels of GM-CSF are indicative of monocyte and granulocyte participation and bone marrow activity; GM-CSF is thought to have an active defensive role in malaria through IL-12 production (Trinchieri, 1998, Mosser and Karp, 1999). It is also involved in the promotion of erythropoiesis (Liehl et al., 1994). The low level of GM-CSF in the UM with Hp1 allele could be indicative of poor monocyte participation. On the other hand, the high level of GM-CSF associated with SMA2-2 group indicates absence of bone marrow suppression and offers a better prognosis for the group. Hence, the claimed bone marrow suppression (Perkins et al., 2011), is dependent on Hp phenotype rather than being restricted to severe forms of malaria as was reported (Stoiser et al., 2000).

In conclusion, our results show that inflammation, oxidative stress and cytokines imbalance in clinical malaria syndromes are divided along the lines of Hp phenotypes and are unlikely to be entirely involved at the same time in malaria pathophysiology. Hp phenotypes in clinical malaria seem to modulate the plasma levels of markers of intravascular haemolysis, oxidative stress, macrophage-associated chemokines and both pro/anti-inflammatory cytokines. The levels of these plasma proteins are likely to explain the differences in malaria pathophysiology; our data suggest that oxidative stress and cytokine imbalance is unlikely in malaria subjects with Hp1 allele with the exception of SMA1-1 while malaria subjects with Hp2 allele with the exception of SMA2-2 have high oxidative stress and cytokine imbalance. Hp phenotypes could indeed modulate the balance between pro/anti-inflammatory cytokines and could partly explain the inter-individual response to inflammatory disease associated with haemolysis.

CHAPTER 6: RESULT 3

6.0 HIGH THROUGHPUT SEQUENCING OF HAPTOGLOBIN (Hp) GENE REVEALS ASSOCIATION BETWEEN Hp SEQUENCE VARIANTS, PLASMA Hp LEVEL AND DISEASE

6.1 Background

Haptoglobin (Hp) phenotypes showed distinct frequencies from one population to another as mentioned in the Introduction. Aside from this, the plasma Hp level differs from one population to another and also varies among normal and diseased persons. The acute phase responsiveness of Hp shows wide differences in inflammatory disease. Furthermore, and as mentioned in the Introduction, different studies have reported association between Hp and disease, and different structural variants of Hp protein have been described (Carter and Worwood, 2007, Bier, 1967, Connell and Smithies, 1959, Quaye et al., 2006, Quaye, 2008). All of these are indications that certain as yet unknown but important sequence variants in the *Hp* gene could be an underlying factor responsible for these variations in Hp frequency, level, structure and association with disease.

The 'cause and effect' relationship between *Hp* gene sequence variants, Hp plasma level, Hp frequency and association with malaria are both unknown and poorly described. Previous studies on the *Hp* gene reveal different structural variants but their effect and clinical association are either poorly defined or not studied at all. Most Hp associated variants in the dbSNP, dbVar and ClinVar databases are designated as having unknown function. Aside from the upstream variant reported as the 'A-61C' no other variant of the *Hp* gene has been

described to be of clinical relevance, even the A-61C at '-61' was wrongly ascribed (Oliviero et al., 1987).

One of the major controversies is that, on one hand ahaptoglobinaemia/hypohaptoglobinaemia (null/low Hp level) have been described as consequences of malaria infection and are used as metrics of effective malaria therapy or prevention and control (Imrie et al., 2012, Imrie et al., 2006), while on the hand they have been described as having a genetic origin.

Plasma Hp levels were measured at enrollment, during convalescence and following patient recovery to ascertain whether the null/low level could be reversed following malaria treatment. Results presented in Chapter 4 indicate that the null/low Hp level in the SMA group is stable all through convalescence to full recovery. Results in Chapter 5 examining Hp phenotypes and Hp plasma protein levels further indicate that stable and low levels of Hp in the SMA group are mostly associated with the Hp1-1 phenotype.

So far, the results indicate that stable and low Hp levels do not recover in all the population, particularly in the SMA group. Based on this observation, we proposed earlier that this stable and low plasma Hp all through convalescence to recovery could be genetic and a predisposing factor for onset of SMA, particularly in SMA1-1, rather than being the consequence of the disease.

To test this idea further, a high throughput sequence analysis of the *Hp* gene, ensuring good coverage beyond the 5'UTR and the supposed promoter region was performed on a MiSeq illumina machine. Reads were aligned to the reference gene using BWA and the data analyzed for the location of variants, as described in the methodology chapter. The aim was to investigate whether or not there is any genetic basis for low/null plasma Hp level and association between *Hp* gene sequence variants and malaria illness within the population.

The results presented here indicate that certain Hp sequence variants are

associated with Hp plasma levels as well as severe malaria. Interestingly, certain deletions at sites distal and proximal to the translational start site in the 5' flanking region of the Hp gene are strongly associated with severe forms of malaria. The result also indicates that sequence variants upstream of the translation start site of the *Hp* gene (Fig. 2.2) are not only important in disease but also in population genetics as well.

6.2 The nomenclature for human gene variants

It is important to understand some of the nomenclature used in the description of sequence variants in accordance with the Human Genome Variation Society (HGVS) recommendations (v2.0).

For example, a sequence variant designated as g.-43A>G means nucleotide A as reference and G as the variant at position 43, counting to the left (5' direction; upstream) of the ATG translational start site, hence the negative sign. The letter 'g' indicates the type of reference sequence used for the description of the variant, which in this case is the genomic reference sequence.

It is important to mention that more than one genotype combination is possible within a given sequence variant. Therefore, we will be using terms such as genotypes for 'a given sequence variant' or simply mentioning the genotypes within a given variant. For example, the possible genotype combinations for g.-43A>G include, HOM_REF, that is homozygous for reference allele A/A (an 'A' in both alleles) at that -43 position; HOM_VAR means homozygous for a variant allele G/G; and HET genotype for the variant is A/G, meaning one allele has the same nucleotide as the reference while the other allele has the variant nucleotide. In the Variant Call Format (.VCF) file this position is assigned a '0' when the nucleotide in one allele is the same as the reference and a '1' for the variant. When there is more than one possible variant at a given position such as g.-

1203GCCC>ACCC,GCCT, the VCF file will assign a '1' for the first variant (ACCC) and a '2' for the second variant (GCCT) at that position, etc. In line with this, A HOM_REF could be designated as 0/0; HOM_VAR as 1/1 and 2/2; HET_VAR as 1/2; and HET as 0/1 and 0/2. Alternatively, the 0/0 or HOM_REF genotype could be represented as g.-1203GCCC/GCCC.

In addition, a g.-2189delT/C is used to designate a single point heterozygous or homozygous deletion at nucleotide position 2189 upstream of the translational start site. A g.-2189delT/C implies either both T and C are deleted or both TT and CC deleted depending on the genotype combination at that position.

6.3 Cluster of variants are present at some regions on Hp gene

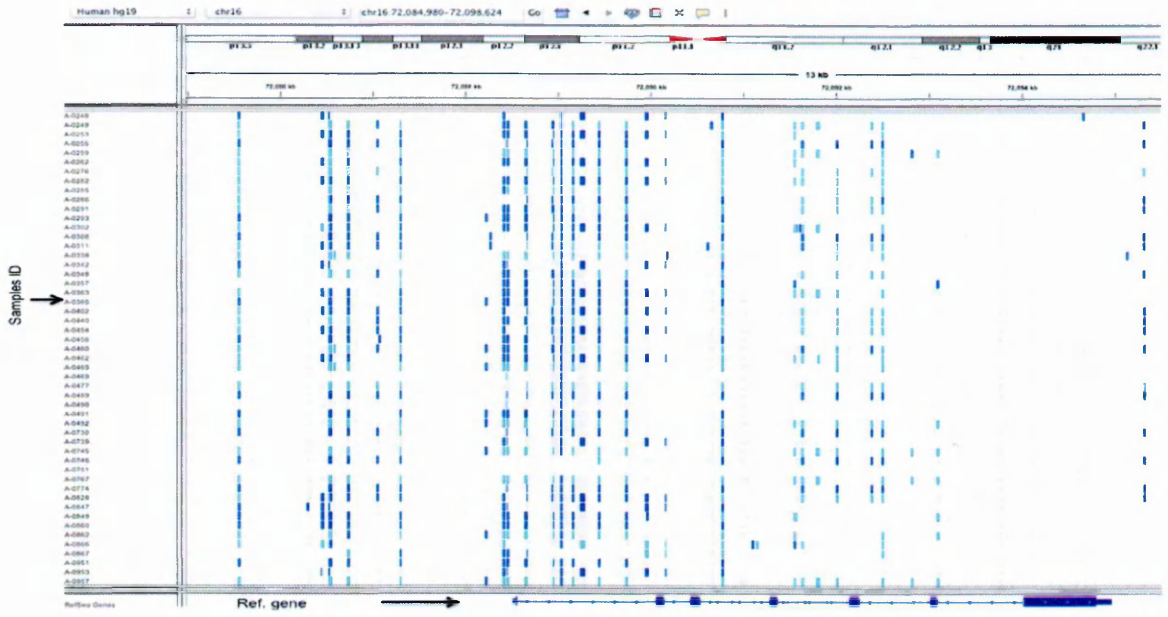
The variant pattern in the *Hp* gene may reveal regions under more genetic pressure. Sequence variants and deletions appear to be more concentrated at certain positions on the gene. Both homozygous and heterozygous deletions are common at both distal and proximal upstream regions (16:72085496-72086460) from the translational start. Clusters of variants are also present at the upstream region from the translational start site at positions 16:72086460-72087324 and 16:72088159-72088467 (Fig. 6.1, Appendix 2). There are also variants at the intronic region. However, sequence variants within the exons are rare (Fig. 6.1).

6.4 Some yet unknown variants on *Hp* gene

The variant call analysis reveals known and unknown sequence variants in the *Hp* gene (Appendix 2, Fig. 6.2). These variants include Single Nucleotide Polymorphisms (SNPs) and insertion and deletions (indels). Some of the variants designated as unknown are sometimes an additional or extended deletion or insertion (indels) at previously described variant positions or the occurrence of an unknown SNP in close proximity to the known (Fig. 6.2), which are sometimes

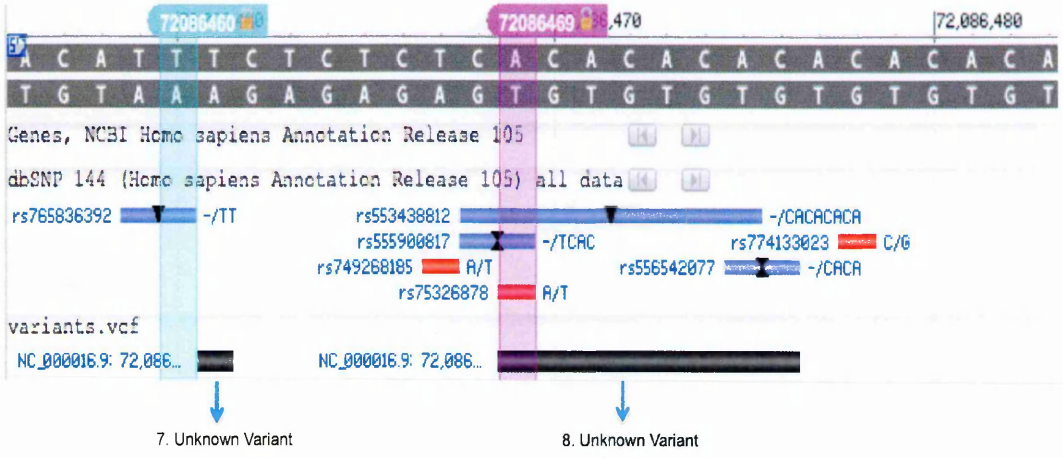
called together as a single variant by the software. In such cases, both the reference and the alternative allele are represented by more than one nucleotide as shown in appendix 2.

Figure 6.1 variants locations on Hp gene



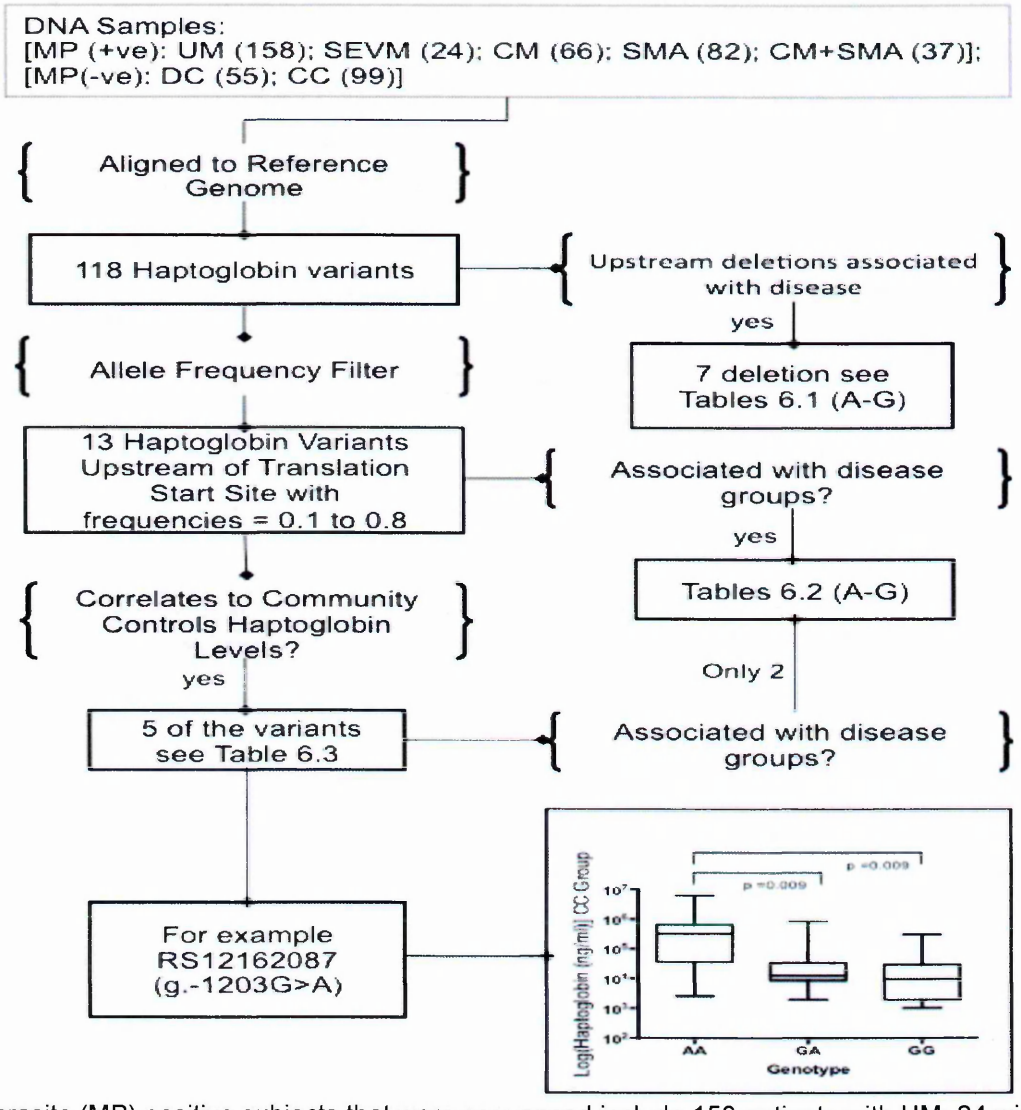
Visualizing the .VCF file in Integrative Genome Viewer (IGV) reveals sites on Hp gene with variations. The light and deep blue dotted colours indicate whether the variation is heterozygous or homozygous with the reference or alternative allele. The arrow on the vertical axis points to sample ID while the horizontal axis points to the reference gene. The variants were visualized on IGV by aligning the .VCF file to the human Hp reference gene on Chr 16:72084980-72098624.

Figure 6.2: Hp sequence variants in the population shows different pattern to known variants in other population



The red colours represent SNP while the purple represent an insertion/deletion (indels). Both have a unique identification number (rs) and correspond to a specific position on *Hp* gene. The nucleotide sequence corresponds to position in the Hp gene where the variants occur. The black bars are an example of an unknown variant, numbered 7 and 8; as was previously described in chapter 2. Variants that extend beyond one or two previously known variants are considered as unknown. Some of the un-known variants identified in the population extend beyond a known variant or across combination of variants. This shows that different deletion parttern could exist within a population.

Figure 6.3. The algorithm for mapping Hp variants to both Hp levels and disease



Malaria parasite (MP) positive subjects that were sequenced include 153 patients with UM; 24 with SEVM; 66 with CM; 82 with SMA and 37 with CM+SMA (Table 3.1) while malaria parasite negative subjects include 55 in the DC and 99 in the CC groups (Table 3.1). Sequences in all of these groups were aligned to the reference genome using BWA. Variants were called with SamTools. Only the variants upstream of the translation start site (proximal and distal promoter) and with population allele frequency ranging from 0.1 to 0.8 were considered out of a total of 118 variants detected (both known and unknown; Appendix 2). Association between Hp variants and levels were considered only in the CC groups while the association between the variants (being SNPs or indels) and disease were computed in the entire disease groups but interpreted relative to the controls (Tables 6.1 and 6.2). The DC group consists of subjects with mostly bacterial infection, particularly typhoid fever. Multiple comparison testing was used to compute for the association between Hp levels and the variants while odds ratio (OR) was computed to determine the association between the variants and disease. Example of one of the variant, g.-1203G>A found to be associated with Hp level is as shown in this figure. The AA genotype (HOMO-VAR) has normal levels while the GA genotype (HET) and the GG genotype (HOMO-REF) have medium and low Hp levels respectively.

6.5 *Hp* gene sequence variants and association with both the disease and plasma Hp levels

The association between Hp variants and levels were only determined in the CC group (Fig. 6.3). Only the variants (indels and SNPs) at both the proximal and the distal region and with the frequency of 0.1-0.8 were analyzed out of the known and unknown 118 variants detected (Appendix 2; Fig. 6.3). This is because the numbers of subjects with variant having frequencies that are less than 0.1 are few and cannot be considered for further analysis.

6.51 Deletions at the distal region upstream from the translational start site protects from severe malaria, particularly SMA.

A strong protection from SMA is associated with the g.-3014delG/T [OR= 0.35 (0.188-0.657), p=0.001]; g.-2949delT/A [OR = 0.44 (0.226-0.870), p=0.018]; g.-2605delT/G [OR =0.40 (0.185-0.872), p=0.021]; g.-2209delG/A [OR=0.34 (0.139-0.779), p=0.011]; g.-2189delT/C [OR=0.34 (0.139-0.779), p=0.011] and the g.-(2050_2052)delT/TTC [OR=0.36 (0.162-0.799), p=0.012]. These deletions protect from SMA to the same magnitude as evident from the OR, CI and the p-values (Table 6.1A-G).

Both the g.-3014delG/T and g.-(2050_2052)delT/TTC significantly [OR=0.37 (0.141-0.976), p=0.045 and OR=0.11 (0.015-0.876), p=0.037] confer protection from respiratory distress and hypoglycaemia; the SEVM group (Tables 6.1A and F).

The g.-2806delTTTTC/T specifically and significantly [OR=0.47(0.218-0.990), p=0.047] confers protection from the CM (Table 6.1E).

The frequencies of these deletions are about 50% lower in severe malaria syndromes compared to the controls, the DC and CC groups (Table 6.1A-G).

There are several homozygous deletions at distal locations upstream of the translation start site and corresponding to positions 16:72085496-72086460. The

deletions occur in close proximity to one another at 3 different positions; -2050 to -2209, -2605 to -2806 and -2949 to -3014 and are about 200bp long (Fig. 6.8) and are identified as g.-3014delG/T, g.-2949delT/A, g.-2806delTTTTC/T, g.-2605delT/G, g.-2209delG/A, g.-2189delT/C and g.-(2050_2052)delT/TTC (Table 6.1 A-G).

Table 6.1 (A-G); Deletions at the upstream region of Hp gene are associated with malaria

A

	g.-3014delG/T (16:72085496)			
Dx	FQ	OR	CI	p-value
CC	0.526			
UM	0.475	0.815	0.491 to 1.352	0.429
SEVM	0.292	0.371	0.141 to 0.976	0.045*
CM	0.493	0.875	0.470 to 1.632	0.676
SMA	0.280	0.352	0.188 to 0.657	0.001*
CM+SMA	0.333	0.451	0.203 to 1.003	0.051
DC	0.491	0.870	0.449 to 1.686	0.680

B

	g.-2949delT/A (16:72085561; rs9924964)			
Dx	FQ	OR	CI	p-value
CC	0.371			
UM	0.278	0.654	0.381 to 1.121	0.123
SEVM	0.208	0.446	0.153 to 1.297	0.138
CM	0.239	0.532	0.265 to 1.067	0.075
SMA	0.207	0.443	0.226 to 0.870	0.018*
CM+SMA	0.222	0.484	0.199 to 1.176	0.109
DC	0.327	0.824	0.410 to 1.656	0.587

C

	g.-2605delT/G (16:72085905)			
Dx	FQ	OR	CI	p-value
CC	0.278			
UM	0.190	0.608	0.335 to 1.103	0.101
SEVM	0.167	0.519	0.162 to 1.657	0.268
CM	0.149	0.455	0.203 to 1.018	0.055
SMA	0.134	0.402	0.185 to 0.872	0.021*
CM+SMA	0.167	0.519	0.194 to 1.385	0.190
DC	0.255	0.885	0.417 to 1.878	0.751

D

	g.-2209delG/A (16:72086301; rs543817830)			
Dx	FQ	OR	CI	p-value
CC	0.247			
UM	0.152	0.545	0.289 to 1.027	0.060
SEVM	0.083	0.277	0.061 to 1.263	0.097
CM	0.134	0.472	0.204 to 1.093	0.080
SMA	0.098	0.329	0.139 to 0.779	0.011*
CM+SMA	0.083	0.277	0.078 to 0.983	0.047*
DC	0.182	0.676	0.296 to 1.544	0.353

Dx= disease groups; FQ = frequency of the deletion; OR = Odds Ratio; CI = Confidence interval. The tables indicate the designated upstream deletions; their frequencies; CI; odds ratio, which is the measure of association between the presence of the genotype and the disease outcome; and p-value within each disease groups. A deletion is designated as being significantly associated with protection or susceptibility if the p-value is less than 0.05 ($p<0.05$) and showed to have lower or higher frequency in the disease compared to the healthy CC respectively.

E

g.-2806delTTTTC/T (16:72085704; rs551013110)				
Dx	FQ	OR	CI	p-value
CC	0.320			
UM	0.222	0.606	0.343 to 1.070	0.084
SEVM	0.167	0.426	0.134 to 1.352	0.148
CM	0.179	0.465	0.218 to 0.990	0.047*
SMA	0.195	0.516	0.258 to 1.032	0.061
CM+SMA	0.167	0.426	0.161 to 1.129	0.086
DC	0.273	0.798	0.384 to 1.658	0.546

F

(g.-2050_2052delT/TTC; 72086460-72086462)				
FQ	OR	CI	p-value	
0.278				
0.177	0.558	0.306 to 1.021	0.058	
0.042	0.113	0.015 to 0.876	0.037*	
0.149	0.455	0.203 to 1.018	0.055	
0.122	0.360	0.162 to 0.799	0.012*	
0.083	0.236	0.067 to 0.833	0.025*	
0.236	0.802	0.374 to 1.723	0.573	

G

g.-2189delT/C (16:72086321; rs112925814)				
Dx	FQ	OR	CI	p-value
CC	0.247			
UM	0.158	0.572	0.305 to 1.072	0.081
SEVM	0.083	0.277	0.061 to 1.263	0.097
CM	0.134	0.472	0.204 to 1.093	0.080
SMA	0.098	0.329	0.139 to 0.779	0.011*
CM+SMA	0.111	0.380	0.122 to 1.186	0.096
DC	0.200	0.760	0.340 to 1.702	0.505

Dx= disease groups; FQ = frequency of the deletion; OR = Odds Ratio; CI = Confidence interval. The tables indicate the designated upstream deletions; their frequencies; CI; odds ratio, which is the measure of association between the presence of the genotype and the disease outcome; and p-value within each disease groups. A deletion is designated as being significantly associated with protection or susceptibility if the p-value is less than 0.05 ($p<0.05$) and showed to have lower or higher frequency in the disease compared to the healthy CC respectively.

6.52 Variants at both distal and proximal regions upstream from the translational start site are associated with malaria

Both the odds ratio (OR) and genotype frequency were computed to determine the association between variants and disease in line with the algorithm (Fig. 6.3). The HOM-VAR (1/1) genotype for the variant, g.-2949T>A significantly predisposes to both CM and SMA (Table 6.2A) while the HET (0/1) genotype for the g.-(1203_1206)GCCC>ACCC,GCCT variant is associated with low risk of SMA (Table 6.2D).

The HET (0/1) for the variants, g.-1976A>T and g.-92TGAC>AGAC,TGAG,TTAC are associated with reduced risk of UM (Tables 6.2B and F). The HET (0/1) for the variant, g.-1976A>T is also associated with a significantly [OR=0.35 (0.168-0.747), p=0.0064] reduced risk of CM while the HOM-VAR (1/1) is associated with an increased risk of CM [OR=2.79 (1.347-5.790), p=0.0057] and fever of bacterial aetiologies (OR=2.17 (1.034-4.566), p=0.041); the DC group (Table 6.2B). Also associated with an increased risk of CM is the HET-VAR (1/2) genotype for the g.-92TGAC>AGAC,TGAG,TTAC variant (Table 6.2F). Furthermore, while the HET genotype for the variant, g.-1452T>C is strongly and uniquely associated with protection from fever of bacterial etiologies, the DC group (Table 6.2C). The HET genotype for g.-283T>C variant is uniquely and significantly [OR=2.17 (1.025-4.580), p=0.042] associated with the DC group (Table 6.2E).

Though the allele frequency (AF) of the variant, g.-49A>C is very low (Table 6.2G), it appear to have an important role in infection. The HOM-REF (0/0) genotype for this variant is associated with an increased risk of both the uncomplicated (UM) and the severe forms (CM and SMA) of malaria while the HET (0/1) genotype is associated with reduced risk of developing both severe and uncomplicated malaria (Table 6.2G). Interestingly, the HET (0/1) genotype for this

variant do not only protect from malaria but appear to be associated with protection from a number of bacterial infection in the DC group (Table 6.2G).

Table 6.2 (A-G) Variants at the upstream region on Hp gene are associated with malaria

A

g.-2949T>A (16:72085561; rs9924964), AF=0.82768												
	0/0				0/1				1/1			
Dx	FG	OR	CI	P-value	FG	OR	CI	P-value	FG	OR	CI	P-value
CC	0.021				0.227				0.412			
UM	0.019	0.919	0.1509 to 5.6029	0.927	0.209	0.900	0.4887 to 1.6576	0.735	0.475	1.288	0.7726 to 2.1461	0.332
SEVM	0.042	2.065	0.1794 to 23.7734	0.561	0.208	0.897	0.3005 to 2.6784	0.846	0.500	1.425	0.5814 to 3.4927	0.439
CM	0.015	0.720	0.0639 to 8.1014	0.790	0.119	0.462	0.1921 to 1.1124	0.085	0.627	2.394	1.2634 to 4.5365	0.0074*
SMA	0.024	1.188	0.1636 to 8.6217	0.865	0.183	0.763	0.3662 to 1.5907	0.471	0.573	1.914	1.0547 to 3.4720	0.0328*
CM+SMA	0.083	4.318	0.6909 to 26.9874	0.118	0.167	0.682	0.2516 to 1.8480	0.452	0.528	1.593	0.7380 to 3.4368	0.236
DC	0.055	2.740	0.4436 to 16.9284	0.278	0.182	0.758	0.3291 to 1.7441	0.514	0.509	1.112	0.5119 to 2.4145	0.789

B

g.-1976A>T (16:72086534; rs7201866), AF=0.83269												
	0/0				0/1				1/1			
Dx	FG	OR	CI	P-value	FG	OR	CI	P-value	FG	OR	CI	P-value
CC	0.021				0.381				0.598			
UM	0.019	0.9194	0.1509 to 5.6029	0.9273	0.259	0.5683	0.3303 to 0.9777	0.0412*	0.677	1.4108	0.8343 to 2.3856	0.1992
SEVM	0.042	2.0652	0.1794 to 23.7734	0.5607	0.292	0.6677	0.2529 to 1.7630	0.4149	0.625	1.1207	0.4463 to 2.8141	0.8083
CM	0.015	0.7197	0.0639 to 8.1014	0.79	0.179	0.3538	0.1676 to 0.7467	0.0064*	0.806	2.7931	1.3474 to 5.7899	0.0057*
SMA	0.037	1.8038	0.2940 to 11.0656	0.5239	0.244	0.5231	0.2732 to 1.0016	0.0505*	0.683	1.4483	0.7812 to 2.6851	0.2396
CM+SMA	0.056	2.7941	0.3786 to 20.6202	0.3137	0.222	0.4633	0.1910 to 1.1239	0.0888	0.722	1.7483	0.7588 to 4.0281	0.1896
DC	0.055	2.7404	0.4436 to 16.9284	0.2779	0.255	0.5537	0.2663 to 1.1514	0.1135	0.764	2.1724	1.0336 to 4.5659	0.0406*

Dx= disease groups; FG = Genotype frequency; OR = Odds Ratio; CI = Confidence interval; AF = population Allele or variant frequency. The tables indicate the genotypes combination associated with a given variant; their frequencies; CI; odds ratio; and degree of significance within each disease groups. A genotype is designated as being significantly associated with protection or susceptibility if $p<0.05$ and showed to have lower or higher frequency in the disease compared to the healthy CC respectively.

C

g.-1452T>C (16:72087058; rs28639994), AF=0.16794												
	0/0				0/1				1/1			
Dx	FG	OR	CI	pvalue	FG	OR	CI	pvalue	FG	OR	CI	pvalue
CC	0.711				0.3093				0.0206			
UM	0.684	0.8765	0.5044 to 1.5231	0.6401	0.2532	0.7571	0.4323 to 1.3258	0.3303	0.0443	2.202	0.4480 to 10.8231	0.3312
SEVM	0.625	0.6763	0.2653 to 1.7242	0.4128	0.3333	1.1167	0.4311 to 2.8922	0.8202	0	0.7796	0.0362 to 16.7719	0.8736
CM	0.746	1.1935	0.5903 to 2.4132	0.6224	0.209	0.5899	0.2844 to 1.2236	0.1563	0.0299	1.4615	0.2007 to 10.6411	0.7079
SMA	0.72	1.041	0.5424 to 1.9979	0.9039	0.2317	0.1563	0.2844 to 1.2236	0.1563	0.0366	1.8038	0.2940 to 11.0656	0.5239
CM+SMA	0.667	0.8116	0.3573 to 1.8435	0.618	0.3056	0.6735	0.3447 to 1.3159	0.2475	0.0278	1.3571	0.1193 to 15.4401	0.8056
DC	0.727	1.0821	0.5172 to 2.2641	0.834	0.2727	0.3175	0.1521 to 0.6625	0.0022*	0.0727	3.7255	0.6597 to 21.0389	0.1365

D

g.-1203G>A (16:72087307), AF=0.65774												
	0/0				0/1				1/1			
Dx	FG	OR	CI	P-value	FG	OR	CI	P-value	FG	OR	CI	P-value
CC	0.082				0.526				0.423			
UM	0.114	1.4304	0.5967 to 3.4285	0.4223	0.405	0.6141	0.3689 to 1.0223	0.0608	0.449	1.1147	0.6690 to 1.8571	0.6768
SEVM	0.125	1.5893	0.3882 to 6.5067	0.5194	0.583	1.2627	0.5113 to 3.1186	0.613	0.250	0.4553	0.1662 to 1.2475	0.126
CM	0.090	1.0943	0.3615 to 3.3122	0.8733	0.478	0.8246	0.4421 to 1.5382	0.5444	0.418	0.9806	0.5218 to 1.8428	0.9515
SMA	0.183	2.614	1.047-6.522	0.039*	0.366	0.5204	0.2854 to 0.9488	0.033*	0.439	1.0689	0.5903 to 1.9356	0.8258
CM+SMA	0.139	1.7944	0.5460 to 5.8970	0.3355	0.389	0.574	0.2632 to 1.2517	0.1628	0.472	1.2221	0.5667 to 2.6352	0.609
DC	0.127	1.6224	0.5546 to 4.7460	0.3769	0.436	0.6983	0.3590 to 1.3584	0.2902	0.509	1.4164	0.7287 to 2.7533	0.3046

Dx= disease groups; FG = Genotype frequency; OR = Odds Ratio; CI = Confidence interval; AF = population Allele or variant frequency. The tables indicate the genotypes combination associated with a given variant; their frequencies; CI; odds ratio; and degree of significance within each disease groups. A genotype is designated as being significantly associated with protection or susceptibility if $p<0.05$ and showed to have lower or higher frequency in the disease compared to the healthy CC respectively.

E

g.-283T>C (72088227; rs5466), AF=0.11905												
	0/0				0/1				1/1			
Dx	FG	OR	CI	P-value	FG	OR	CI	P-value	FG	OR	CI	P-value
CC	0.814				0.196				0.021			
UM	0.772	0.7089	0.3757 to 1.3375	0.2881	0.203	1.0426	0.5531 to 1.9655	0.8974	0.013	0.609	0.0844 to 4.3954	0.6228
SEVM	0.750	0.6835	0.2377 to 1.9656	0.4802	0.208	1.0803	0.3577 to 3.2631	0.891	0.000	0.7796	0.0362 to 16.7719	0.8736
CM	0.806	0.9464	0.4283 to 2.0915	0.8918	0.179	0.8957	0.4021 to 1.9952	0.7875	0.000	0.283	0.0134 to 5.9891	0.4176
SMA	0.768	0.7555	0.3660 to 1.5595	0.4483	0.220	1.1546	0.5595 to 2.3827	0.6973	0.000	0.2315	0.0110 to 4.8918	0.3472
CM+SMA	0.722	0.5924	0.2430 to 1.4441	0.2495	0.250	1.3684	0.5531 to 3.3855	0.4974	0.028	1.3571	0.1193 to 15.4401	0.8056
DC	0.727	0.6076	0.2775 to 1.3304	0.2127	0.350	2.1667	1.0249 to 4.5802	0.042*	0.000	0.3441	0.0162 to 7.2987	0.4937

Dx= disease groups; FG = Genotype frequency; OR = Odds Ratio; CI = Confidence interval; AF = population Allele or variant frequency. The tables indicate the genotypes combination associated with a given variant; their frequencies; CI; odds ratio; and degree of significance within each disease groups. A genotype is designated as being significantly associated with protection or susceptibility if $p<0.05$ and showed to have lower or higher frequency in the disease compared to the healthy CC respectively.

F

g.-92TGAC>AGAC,TGAG,TTAC (16: 72088418), AF=0.43919												
	0/0				0/1				1/1			
Dx	FG	OR	CI	P-value	FG	OR	CI	P-value	FG	OR	CI	P-value
CC	0.165				0.454				0.196			
UM	0.158	0.9516	0.4794 to 1.8889	0.8872	0.323	0.5741	0.3411 to 0.9663	0.0367*	0.209	1.0838	0.5764 to 2.0377	0.8027
SEVM	0.125	0.7232	0.1926 to 2.7161	0.6312	0.375	0.7227	0.2886 to 1.8099	0.4881	0.083	0.3732	0.0807 to 1.7269	0.2073
CM	0.060	0.3214	0.1024 to 1.0091	0.0518	0.328	0.5889	0.3081 to 1.1257	0.1092	0.179	0.8957	0.4021 to 1.9952	0.7875
SMA	0.110	0.6241	0.2600 to 1.4984	0.2915	0.341	0.6246	0.3405 to 1.1458	0.1284	0.207	1.0737	0.5161 to 2.2335	0.8491
CM+SMA	0.194	1.222	0.4567 to 3.2699	0.6897	0.278	0.4633	0.2017 to 1.0643	0.0698	0.222	1.1729	0.4618 to 2.9791	0.7373
DC	0.182	1.125	0.4713 to 2.6855	0.7908	0.345	0.6357	0.3206 to 1.2607	0.1947	0.182	0.9123	0.3903 to 2.1325	0.8322
	0/2				1/2				2/2			
Dx	FG	OR	CI	P-value	FG	OR	CI	P-value	FG	OR	CI	P-value
CC	0.124				0.093				0.010			
UM	0.114	0.9107	0.4181 to 1.9839	0.8139	0.120	1.3365	0.5788 to 3.0862	0.4969	0.038	3.6923	0.4378 to 31.1404	0.2299
SEVM	0.208	1.864	0.5868 to 5.9211	0.2909	0.125	1.3968	0.3477 to 5.6118	0.6376	0.042	4.1739	0.2516 to 69.2561	0.3188
CM	0.119	0.9605	0.3699 to 2.4941	0.9339	0.209	2.5828	1.0458 to 6.3787	0.0397*	0.060	6.0952	0.6658 to 55.8003	0.1096
SMA	0.146	1.2143	0.5136 to 2.8708	0.6583	0.122	1.358	0.5237 to 3.5216	0.529	0.049	4.9231	0.5392 to 44.9502	0.1578
CM+SMA	0.083	0.6439	0.1707 to 2.4287	0.5158	0.167	1.9556	0.6425 to 5.9518	0.2376	0.056	5.6471	0.4961 to 64.2830	0.163
DC	0.091	0.7083	0.2357 to 2.1284	0.539	0.127	1.4259	0.4997 to 4.0687	0.5071	0.073	7.5294	0.8198 to 69.1532	0.0744

G

g.-49A>C (16:72088461; rs5471), AF=0.13953												
	0/0				0/1				1/1			
Dx	FG	OR	CI	p-value	FG	OR	CI	p-value	FG	OR	CI	p-value
CC	0.629				0.340				0.021			
UM	0.753	1.801	1.0409 to 3.1154	0.0354*	0.222	0.552	0.3142 to 0.9694	0.0386*	0.006	0.303	0.0271 to 3.3820	0.332
SEVM	0.667	1.180	0.4595 to 3.0319	0.731	0.292	0.799	0.3011 to 2.1178	0.651	0.042	2.065	0.1794 to 23.7734	0.561
CM	0.806	2.452	1.1786 to 5.0989	0.0164*	0.164	0.381	0.1762 to 0.8235	0.0141*	0.015	0.720	0.0639 to 8.1014	0.790
SMA	0.768	1.957	1.0135 to 3.7782	0.0455*	0.183	0.434	0.2156 to 0.8743	0.0195*	0.024	1.188	0.1636 to 8.6217	0.865
CM+SMA	0.750	1.771	0.7495 to 4.1820	0.193	0.222	0.554	0.2273 to 1.3507	0.194	0.028	1.357	0.1193 to 15.4401	0.806
DC	0.709	1.439	0.7052 to 2.9346	0.318	0.164	0.379	0.1657 to 0.8691	0.0219*	0.055	2.740	0.4436 to 16.9284	0.278

Dx= disease groups; FG = Genotype frequency; OR = Odds Ratio; CI = Confidence interval; AF = population Allele or variant frequency. The tables indicate the genotypes combination associated with a given variant; their frequencies; CI; odds ratio; and degree of significance within each disease groups. A genotype is designated as being significantly associated with protection or susceptibility if $p<0.05$ and showed to have lower or higher frequency in the disease compared to the healthy CC respectively.

6.53 Sequence variants at proximal and distal regions upstream of translation start site are associated with plasma Hp levels

Multiple comparison testing was performed in order to determine the association between the levels of plasma Hp associated with the different genotypes in the CC groups as described in fig. 6.3.

Plasma Hp levels are associated with the g.-1203G>A, g.-1955T>C, g.-(1811_1806)GCCTGA>ACCTGG, g.-49A>C and g.-43A>G variants (Table 6,3). Normal plasma Hp levels are found in >70% of the population that are HOM-VAR for g.-1203G>A, g.-1955T>C and g.-(1811_1806)GCCTGA>ACCTGG and HOM-REF for g.-49A>C and g.-43A>G (Fig. 6.3; Table 6.3). Those that are HET for these variants have lower than normal plasma Hp levels. On the other hand, presence of HOM-VAR for g.-49A>C and g.-43A>G and HOM-REF g.-1955T>C and g.-(1811_1806)GCCTGA>ACCTGG and including either the homozygous or heterozygous deletions at these positions are associated with low plasma Hp level (Table 6.3).

Both the g.-1203G>A and the g.-49A>C also show association with disease. However, the g.-49A>C showed association with the UM, CM, SMA and DC and is not restricted to only malaria syndromes while the g.-1203G>A is restricted to only the SMA (Tables 6.2D and G). While the g.-1203G/G confers susceptibility to SMA, the g.-1203G/A confers protection, considering their frequencies in the disease groups compared to the CC and the OR (Table 6.2D; Table 6.3). The g.-49A/A which is associated with high levels appears to confer susceptibility to malaria while the g.-49A/C which is associated with suboptimal plasma Hp levels appear to confer protection to malaria and bacteria infection (Table 6.2G; Table 6.3).

Table 6.3 Genotypes for an Hp gene variants are associated with Hp protein level

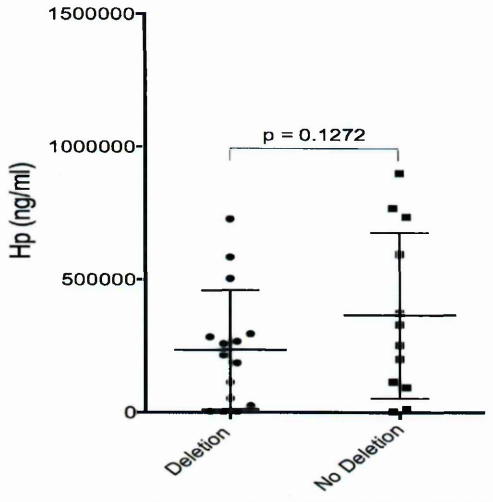
Sequence Variants	Levels		
	Normal	Medium	Low
g.-1203G>A (16:72087307)	HOM-VAR	HET	HOM-REF, Del
g.-1955T>C (16:72086555; rs7203426)	HOM-VAR	HET	HOM-REF, Del
g.-(1811_1806)GCCTGA>GCCTGG, ACCTGG(16:72086741)	HOM-VAR	HET	HOM-REF, Del
g.-49A>C (16:72088461; rs5471)	HOM-REF	HET	HOM-VAR, Del
g.-43A>G (16:72088467; rs5472)	HOM-REF	HET	HOM-VAR, Del

The associations of these genotypes with levels are independent of disease groups and were only confirmed in the healthy CC using the algorithm in Fig. 6.3. The rs7203426; rs5471 and rs5472 correspond to SNPs ID. Previously unknown variant have no SNP ID.

6.54 Deletions at the proximal upstream sites on *Hp* gene do not affect plasma Hp levels

The g.-3014delG/T, g.-2949delT/A, g.-2806delTTTTC/T, g.-2605delT/G, g.-2209delG/A, g.-2189delT/C and g.-(2050_2052)delT/TTC deletions covering 16:72085496-72086460 (Table 6.1A-G) as earlier mentioned do not affect plasma Hp level. Hp levels are not associated with any of these deletions found at positions 16:72085496-72086460 (Fig. 6.4). Plasma Hp levels in individuals with any of these deletions are not statistically different to those without any of the deletions (Fig. 6.4). While the deletions are associated with severe malaria, they do not appear to be associated with plasma Hp levels. However, long or short homozygous or heterozygous deletions downstream of positions 16:72085496-72086460 are more associated with plasma Hp levels rather than malarial illness (Table 6.3).

Figure 6.4 Upstream deletion spanning 16:72085496-72086460 do not affect plasma Hp level.



deletions at 16:72085496-72086460
are not associated with plasma Hp levels

Mann Whitney test was used to compare between the two groups. The data points in the graph consist of 15 subjects in the CC group with either of the deletions and 12 subjects without the deletions.

6.55 The genotypes that are associated with Hp plasma levels also determine Hp phenotype susceptibility to SMA.

The SMA1-1 group have lower frequencies of 1/1 genotypes from the g.-1203G>A; g.-1955T>C and g.(-1811_-1806)GCCTGA>ACCTGG variants that are associated with normal plasma Hp levels and a higher frequency of 0/0; A/A genotype from the variant, g.-43A>G also known to be associated with normal plasma Hp level (Table 6.4).

Unlike the SMA1-1 group, the SMA2-2 (SMA group with Hp2-2) have higher frequencies of the variants that are associated with normal plasma Hp levels compared to the CC; the frequencies of 1/1 genotypes for the g.-1203G>A; g.-1955T>C and g.(-1811_-1806)GCCTGA>ACCTGG; 0/0 genotype for the g.-43A>G are higher in the SMA2-2 group. The frequencies of the 0/1 genotypes across the entire variants with Hp2-2 are lower in the SMA compared to the CC (Table 6.4).

While the entire 0/1 genotypes are of lower frequencies in the SMA2-2 group compared to those of the CC group, only the 0/1 genotype from the g.-49A>C shows lower frequency compared to that of the CC group in the Hp1-1 subjects with SMA (SMA1-1) (Table 6.4).

Table 6.4 Genotypes from variants that are associated with plasma Hp levels show different frequencies within a given Hp phenotype in SMA subjects

HP Phenotype	Sequence Variants	CC	SMA	CC	SMA	CC	SMA
		Genotypes					
		1/1	1/1	0/1	0/1	0/0	0/0
HP1-1	g.-1203G>A	0.56	0.43	0.4	0.348	0.04	0.174
	g.-1955T>C	0.600	0.435	0.360	0.435	0.04	0.087
	g.(-1811_-1806)GCCTGA>ACCTGG	0.560	0.435	0.320	0.391	0.04	0.174
	g.-49A>C	0.000	0.000	0.120	0.043	0.88	0.957
	g.-43A>G	0.480	0.435	0.440	0.391	0.08	0.174
HP2-1	g.-1203G>A	0.396	0.45	0.479	0.375	0.125	0.175
	g.-1955T>C	0.438	0.400	0.417	0.425	0.104	0.175
	g.(-1811_-1806)GCCTGA>ACCTGG	0.396	0.425	0.479	0.375	0.125	0.175
	g.-49A>C	0.021	0.000	0.396	0.275	0.583	0.725
	g.-43A>G	0.146	0.175	0.604	0.475	0.250	0.350
HP2-2	g.-1203G>A	0.300	0.400	0.670	0.400	0.037	0.200
	g.-1955T>C	0.296	0.400	0.667	0.400	0.000	0.200
	g.(-1811_-1806)GCCTGA>ACCTGG	0.259	0.400	0.667	0.400	0.037	0.200
	g.-49A>C	0.037	0.133	0.407	0.267	0.556	0.533
	g.-43A>G	0.074	0.067	0.593	0.400	0.333	0.533

The genotypes found to be associated with plasma Hp levels are of different frequencies within a given Hp phenotypes in SMA subjects. Shaded areas represent genotype frequency relative to the CC.

6.6 Long *Hp* gene deletions

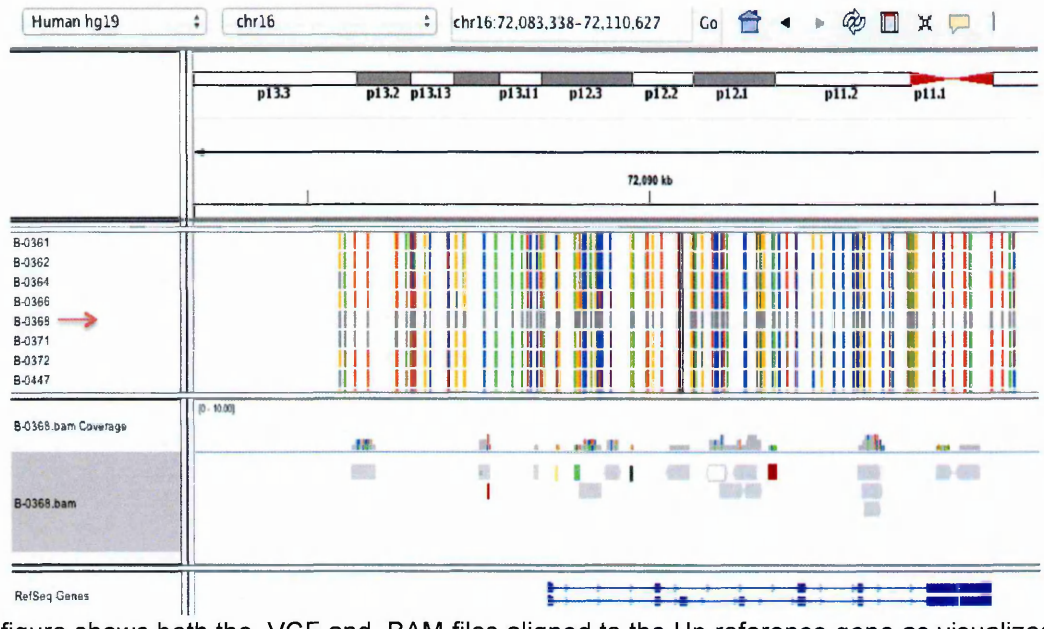
There is a rare homozygous deletion of the entire *Hp* gene in the population. A homozygous deletion of the entire *Hp* gene (Fig. 6.5) was seen in one (B-0368) out of 523 samples. Both the VCF and BAM files mapped to the reference gene in the IGV indicate a complete deletion of both alleles in the patient. The deletion in the VCF file is indicated as grey colour in the direction of the arrow while that of the BAM file is shown by the zero coverage and the absence of reads (Fig. 6.5).

Another deletion of both alleles spanning from a distant upstream position of the translational start site to position beyond exon 6 of Hp2 allele or exon 4 of Hp1 allele (16:72093742) but not including exon 7 of Hp2 and exon 5 of Hp1 allele was seen in one sample, A-0751 (Fig. 6.6). Both B-0368 and A-0751 have null plasma Hp levels, lower than normal plasma HPx levels but normal plasma fHb levels.

Another long heterozygous deletion (deletion on one allele) as well as short homozygous deletions occur from the 5'UTR to the intronic region between exon 6 and 7 and spanning region 16:72088231-72093725 were present in only sample E-0988 (Fig. 6.7), a patient presenting with gastroenteritis as one of the DC group. The plasma Hp and HPx levels in this sample are normal, 0.5g/L and 1.1g/L but with a very high plasma fHb level unlike in samples B-0368 and A-751.

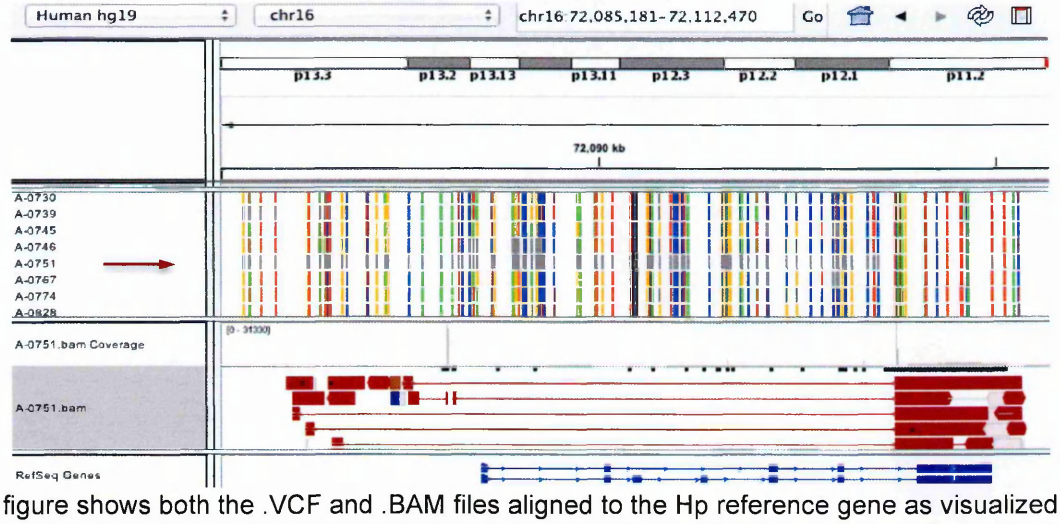
The Hp phenotypes and genotypes for both B-0368 and A-0751 could not be determined by western blot and PCR methods. Aside of these 3 samples, entire long deletions of the Hp gene appears to be rare in the population. Samples with very good coverage and without any of these deletions are as shown (Fig. 6.8).

Figure 6.5: Homozygous deletion of entire Hp gene



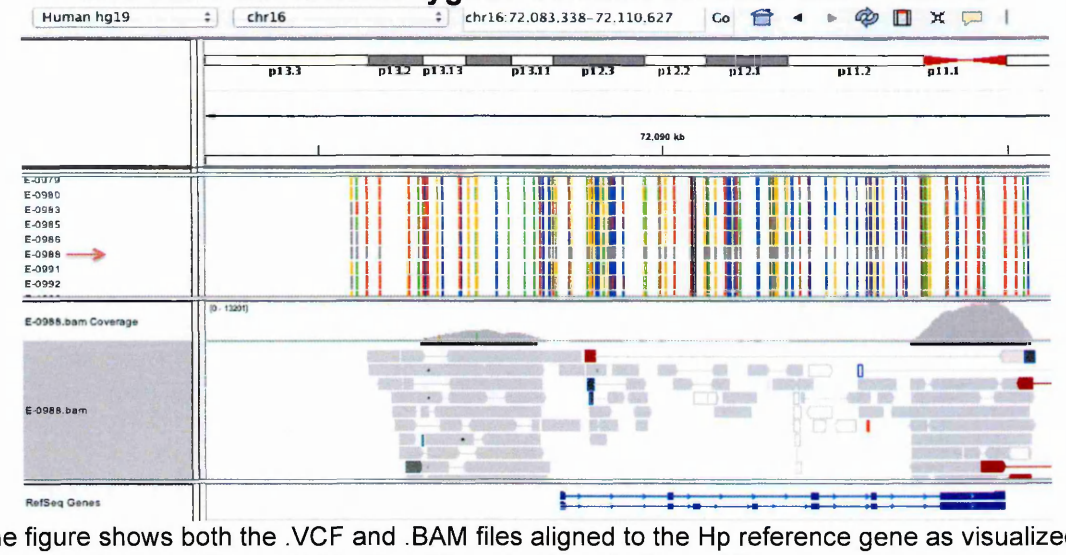
The figure shows both the .VCF and .BAM files aligned to the Hp reference gene as visualized in Integrative Genome Viewer (IGV). The different colours indicate different variants in the subjects as found in the .VCF file. The gray colour in the direction of the arrow indicates complete deletion of the gene in the subject, B-0368. Below the .VCF file is the .BAM file uploaded for the same subject and showing complete disappearance of the coverage and reads, which indicates complete deletion across the entire Hp length.

Figure 6.6: Homozygous deletion of Hp gene from the promoter to the intron between exon 5 and 6



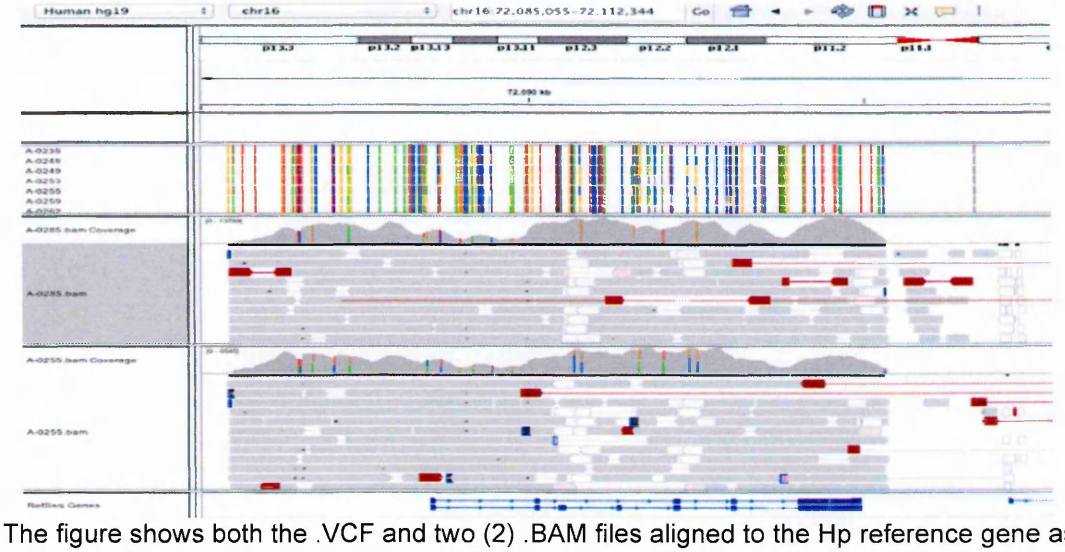
The figure shows both the .VCF and .BAM files aligned to the Hp reference gene as visualized in Integrative Genome Viewer (IGV). The different colours indicate different variants in the subjects as found in the .VCF file. The gray colour in the direction of the arrow indicates deletion of the gene that extends from the promoter to the intronic region just before the last exon. Below the .VCF file is the .BAM file and showing complete deletion of the reads and zero coverge. However, a very short region in the promoter and the last exon was spared as indicated by the small read coverage areas.

Figure 6.7 A heterozygous deletion on Hp gene from the promoter to exon 5 and other short homozygous deletion between exon 1 and 5



The figure shows both the .VCF and .BAM files aligned to the Hp reference gene as visualized in Integrative Genome Viewer (IGV). The different colours indicate different variants in the subjects as found in the .VCF file. The gray colour in the direction of the arrow indicates deletions. The disappearance of the reads/coverage between the promoter and the last exon as shown in the .BAM file is indicative of the deletion. Looking at the .BAM file, it is also obvious that there are some few reads in-between the promoter and the last exon, indicating a likely heterozygous deletion (deletion on one allele).

Figure 6.8 A typical example of reads alignment without any deletion in the promoter and the entire Hp gene



The figure shows both the .VCF and two (2) .BAM files aligned to the Hp reference gene as visualized in Integrative Genome Viewer (IGV). The different colours indicate different variants in the subjects as found in the .VCF file. There are no deletions in these subjects; the .BAM files have both good coverage and overlapping reads across the entire Hp region.

6.7 Population associated Hp sequence variants

Some variants in the Hp gene are found to be common in the entire population such as the HET; CT/C (rs576218458) SNP occurring at position 16:72089044-72089045. The occurence of this SNP is independent of the disease, race or individual variations. The entire population also has a 'slow' and a 'fast' (f-s) but not f-f or/and s-s sub-phenotypes of Hp (Connell et al., 1966, Koch et al., 2003).

6.8 Discussion

The study of human genetic variation has both evolutionary and health significance. For example, Single Nucleotide Polymorphisms (SNPs) have been reported to affect protein function and level (Griffiths et al., 2008). Genetic variations are associated with diversity; susceptibility to diseases; individuality and response to medicine (Shastry, 2002).

Results from the previous chapters have indicated that low plasma Hp levels are likely to be of genetic aetiology. The *Hp* gene appears to have been very prone to genetic pressure; malaria was reported to be responsible for the copy number variation (CNV) in the Hp gene that gave rise to the Hp2 allele (Rodriguez et al., 2012). As a result, the Hp2 allele is generally considered to be protective from malaria. The frequency of Hp2 allele is lower in malaria patients compared to Hp1 as we had observed and as was reported (Atkinson et al., 2007, Elagib et al., 1998). However, the result in Chapter 5 shows that the Hp frequencies in the control groups (DC and CC) are of the same trend and not different to the malaria groups. Hence, we earlier suggested that the *Hp* gene could be under selective pressure for something other than malaria but could confer a selective advantage or disadvantage to malaria and other diseases. Whatever the case might be, variants in the *Hp* gene play some roles in malaria.

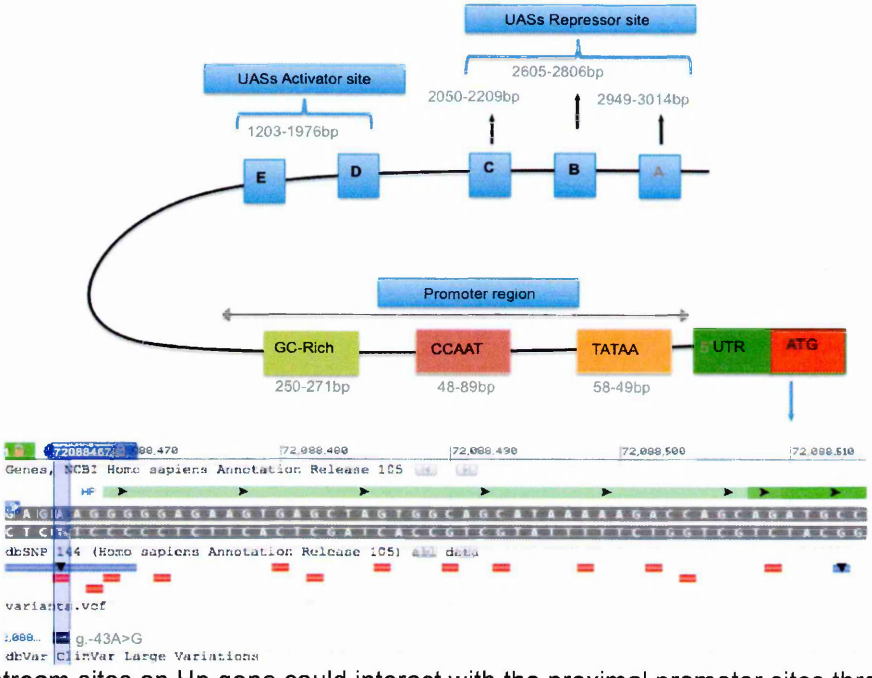
The result shows that sequence variants at the distal and proximal regions upstream from the translational start site of the *Hp* gene are associated with both protection and susceptibility to malaria syndromes and do indicate the roles of *Hp* gene in malaria. Hp sequence variants at both the distal and proximal upstream region, be it indels or SNPs are strong determinants as to whether a child infected with *Plasmodium falciparum* could go on to develop either of the severe forms of malaria.

The deletions at the distal upstream regions are strongly associated with protection from SMA while variant-associated genotypes have been shown to either predispose or protect from different malaria syndromes as well as mild and severe bacterial infections, which indicates that the *Hp* gene could also confer selective advantage or disadvantage to other diseases aside from malaria.

We propose that the distal regions of the *Hp* gene where the deletions occur are spatially close and could interact with the promoter due to DNA looping (Fig. 6.9) that could possibly allow interaction between the proximal promoter and the distal region through DNA binding proteins such as transcription factors and could be important in gene expression.

The close proximity of the 3 different 200bp long regions of deletion also suggests that the sites might have some regulatory functions as proposed in the model (Fig. 6.9). Regions distal to the translational start site are reported to contain some cis-acting regulatory sequences called upstream activation sequences (UASs) or enhancers (Fig. 6.9) that could interact with the promoter-proximal elements (Griffiths et al., 2008). These UASs are thought to be about 200bp long in immunoglobulin genes and could occur in more than a single position (Cooper, 2000, Griffiths et al., 2008) as has also been observed with the Hp gene (Fig. 6.9). Furthermore, the UASs are thought to contain clusters of binding sites for more specific translational factors (Cooper, 2000, Griffiths et al., 2008) as well as some negative and positive regulatory elements such as repressors and activators of translation (Cooper, 2000).

Figure 6.9. The looping model to illustrate possible interaction between the distal and proximal promoter sites on Hp gene



Distal upstream sites on Hp gene could interact with the proximal promoter sites through DNA looping. This looping mechanism could bring both the distal and proximal promoter sites in close proximity. As a result, proteins that are capable of binding to the promoter could also interact with the distal sites. These proteins could either activate or prevent gene expression on binding to the activator or the repressor sites on the gene. We have earlier shown that a deletion of about 200bp long were found at 3 sites at the distal upstream region that correspond to 2050 to 2209, 2605 to 2806 and 2949 to 3014 and designated as C, B and A respectively. These distal upstream deletions were shown to protect from severe malaria (Tables 6.1A-G). As a result, we propose that certain protein-DNA interactions are likely at these designated sites and could enhance disease severity had the deletion not occurred. We considered these regions to have some possible repressor function, as the deletions are likely to prevent certain protein-DNA associations that could enhance disease severity. On the other hand, variants at position designated as D and E protect from both mild and severe forms of malaria (Fig. 6.2B and D). We again proposed that certain interactions at these positions are likely to activate the observed protection. Hence, both D and E are designated as activator sites. The ATG, 5' UTR, TATAA, CCAAT box and the GC-Rich elements are as show in the figure. The Hp sequences corresponding to the ATG, the TATAA box, CCAAT box and the GC-rich sites are as shown in this figure. The proximal promoter region that could possibly interact with the activator and repressor site is as indicated by the horizontal arrow.

In line with the above and the fact that there are normal plasma Hp levels despite these deletions, we proposed that certain DNA-protein interactions at those 3 regions of deletion (Fig. 6.9) are involved in enhancing disease severity/resolution through as yet unknown mechanism(s). Therefore, the deletions confer protection to the severe forms of malaria by preventing such interactions. Example of such possible interaction is that of Hp and prostaglandins that was reported to elicit harmful oxidation processes (Lange, 1992). The protection or susceptibility associated with the observed genotypes might be due to some kind of interactions at the upstream regions of the gene.

The *Hp* gene promoter lies within -43 to -400 nucleotides upstream of the translation start site (Pelletier et al., 1998, Lee et al., 2002, Oliviero et al., 1987) and some of the variants shown to have association with the disease and plasma Hp levels are within these regions, which are also thought to be the binding sites for hormones and cytokines released during acute phase response (Quaye, 2008).

The CCAAT sequence within the Hp promoter is required for the activation of *Hp* gene expression (Oliviero et al., 1987, Pelletier et al., 1998, Lee et al., 2002). Induced mutations at the promoter region have been reported to reduce plasma Hp level. Furthermore, a SNP within the promoter has also been reported to decrease the Hp responsiveness to IL-6 (Grant and Maeda, 1993). Therefore, it is more likely that the effect of these promoter associated sequence variants on plasma Hp levels could be tightly linked to their ability to enhance or inhibit gene expression.

Genotypes associated with Hp levels could determine the susceptibility of Hp phenotypes to SMA. Some genotypes that are associated with normal Hp levels are of lower frequency in the SMA1-1 and 2-1 groups compared to the CC such that normal Hp levels are rarely found in these groups while genotypes that are associated with normal Hp level are of higher frequencies in the SMA2-2 group

compared to the CC. We have earlier shown that while the Hp levels recover in the SMA subjects with Hp2-2, levels do not recover in the SMA subjects with both Hp2-1 and 1-1.

While we suggested that the low levels of Hp could be a risk factor for onset of SMA, the presence of the variant, g.-49A>C, could change the entire pathological equation between Hp levels and disease. Though low Hp level is a risk factor for onset of SMA, it does not appear so in individuals with the g.-49A/C variant, which is shown earlier to be associated with suboptimal Hp levels. On the other hand, high plasma Hp does not appear to protect from SMA in individuals with the g.-49A/A wild type. While the g.-49A/A is associated with high plasma Hp level it significantly predisposes to malaria; UM [OR=1.80(1.041-3.112), p=0.0035]; CM [OR=2.45(1.179-5.99), p=0.016] and SMA [OR=1.96(1.014-3.778), p=0.045] and its frequency is higher in the SMA group with Hp1-1 than Hp2-2. The g.-49A/C variant is observed to be associated with low plasma Hp and protection to malaria; UM [OR=0.55(0.314-0.969), p=0.0386]; CM [OR=0.38(0.176-0.824), p=0.014]; SMA [OR=0.434(0.216-0.874), p=0.02] as well as other bacterial infections; the DC group [OR=0.38(0.166-0.869), p=0.022]. A previous report that low Hp levels are associated with protection from malaria (Cox et al., 2007) could be due to the effect of the g.-49A>C variant.

This genetic effect and the differences associated with Hp levels within the major Hp phenotypes might be responsible for the different responses elicited in the malaria groups by individuals with different Hp phenotypes as mentioned in the previous chapter.

The variant, g.-49A>C is the same as the 'A-61C' that was previously reported (Oliviero et al., 1987, Cox et al., 2007) because the nucleotide sequence flanking the g.-49A>C is the same as those around the A-61C position (Oliviero et al., 1987, Cox et al., 2007). Aside from this g.-49A>C that has been wrongly

reported as the A-61C none of the variants that we have shown to be associated with plasma Hp level have been reported.

Our result agree with the previous observation that the g.-49A>C (A-61C) is associated with reduced plasma Hp level (Cox et al., 2007).

While low/null levels of plasma Hp could occur as a consequence of haemolysis (Delanghe et al., 1998b), we have established that a situation whereby the level remains low following treatment and through convalescence is rather due to genetic factors.

The controversy regarding the aetiology of hypohaptoglobinaemia (low) and ahaptoglobinaemia (null) has not been properly addressed; sequence variants such as SNPs and short indels appear to be the major cause of hypo/ahaptoglobinaemia in this population rather than the long heterozygous and homozygous deletions on *Hp* gene that was previously reported (Koda et al., 2000, Koda et al., 1998b) Different variants of the *Hp* gene could be responsible for ahaptoglobinaemia and hypohaptoglobinaemia in different populations as evident from this study and the previous report in Ghana (Teye et al., 2003, Teye et al., 2004).

Long homozygous and heterozygous deletions of the Hp gene as previously described (Koda et al., 2000, Koda et al., 1998b) are found in only two out of the 519 subjects that were successfully sequenced. Both heterozygous and/or homozygous deletions of an entire Hp gene are rare in African populations (Koda et al., 2000). This long homozygous deletion of the *Hp* gene observed in 2 out of 519 patients in this population do not affect the removal of fHb from circulation. The reduced levels of HPx in these individuals are likely to indicate its role in the removal of fHb from the circulation in the absence of Hp. On the other hand, the high plasma fHb level in the face of normal Hp and HPx levels in a patient with heterozygous deletion from exon 1 to 6 alongside other short deletions

of the intact allele indicate that the binding sites for fHb may have been deleted on the *Hp* gene. This implies that certain short deletions in the *Hp* gene that might not affect plasma Hp level could affect its ability to scavenge fHb.

The variants described in this study are different from those reported in previously in Ghana (Teye et al., 2003, Teye et al., 2004) and other populations (Koda et al., 2000, Koda et al., 1998b). Hence, the *Hp* gene appears to be subject to different genetic pressure in different populations. These differences in genetic pressure with geographical locations could partly explain the presence of some of the unknown variants that are seen in this population as well as the marked differences in the frequencies of Hp phenotypes in different geographical locations. Furthermore, aside the role of *Hp* gene in disease, it could be of importance in population genetics since some of the variants are common to the entire population.

In this study we show that some yet unknown variants of the *Hp* gene are associated with protection and susceptibility to malaria, whether or not they are associated with plasma Hp levels. Furthermore, the genotypes that are associated with Hp plasma levels could also determine the susceptibility rate of Hp phenotypes to SMA. The *Hp* gene could be useful as a promising genetic marker for studying disease susceptibility and/or protection among individuals within a given population and it have been implicated in several diseases. Though the results of some mechanistic studies are already in line with our observation; there is a need to carry out a more mechanistic study to investigate some of the observations in this association-based study. Associations between Hp variant and both Hp levels and disease need to be validated in other populations.

CHAPTER SEVEN: RESULT 4

7.0 POTENTIAL BIOMARKERS FOR CLINICAL MALARIA SYNDROMES

7.1 Background

As mentioned in Chapter 3, *P. falciparum* malaria illness is associated with different clinical presentations. Accurate diagnosis to differentiate between these malaria syndromes and from other causes of fever is important for effective patient management. Aside from general administration of anti-malarial drugs, each malaria syndrome requires different patient care. Malaria syndromes overlap with several illnesses. For example, CM has been defined as generalized convulsion in the presence of asexual forms of the *P. falciparum* parasite. However, in a malaria holoendemic region, there are other causes of convulsion such as meningitis and one could not completely rule out the presence of malaria parasites in such subjects (Aipit et al., 2014). There is therefore the need for a specific biomarker for CM to differentiate it from other causes of convulsion in malaria holoendemic regions.

Furthermore, most fever-associated illnesses have been mistaken and treated as malaria (Moreno-Caballero et al., 2016, Decuypere et al., 2016), particularly in non-malarial paediatric fever (Al-Harthi, 2015) and could place higher demand on antimalarial therapy leading to drug resistance. It is therefore important to search for biomarkers that could differentiate among the malaria syndromes and between malaria syndromes and other causes of fever.

Effective diagnosis alongside appropriate treatment remains the main malaria control strategy beside any effective preventive measures. The most commonly recommended methods of malaria diagnosis are microscopy, Malaria Rapid Diagnostic Test (MRDT) and clinical observation.

Despite these recommendations, the diagnosis of clinical malaria is still complicated as a result of its different clinical manifestations; symptom overlap with other causes of fever; concurrent malaria parasite and bacteria co-infection and the need for trained specialist in the areas of microscopy and molecular techniques.

There are also some downsides to the use of MRDTs that are designed to detect Parasite Histidine-rich Protein2 (pHRP2) and/or *Plasmodium* Lactate Dehydrogenase (pLDH), (Mbabazi et al., 2015). For example, HRP2 can persist in circulation for several weeks after parasite clearance (Kyabayinze et al., 2008, Mbabazi et al., 2015, Swarthout et al., 2007). Therefore, the use of MRDT for HRP2 detection and diagnosis is not effective in regions where malaria is holoendemic and for patients with recent malaria episodes, which may lead to false positive results (Mbabazi et al., 2015). On the other hand, the pLDH-based assay can provide a false negative result at low parasite density (Mbabazi et al., 2015). More importantly, both pHRP2 and pLDH target parasite products, and are not useful for differentiating the onset of clinical malaria.

Microscopic examination of giemsa stained blood is useful for detection and in differentiating between parasites species or stages in the life cycle such as distinguishing between clinically important asexual forms of the parasite and gametocytes, and for quantifying parasite density. However microscopy cannot be used to differentiate among the clinical malaria syndromes. Both molecular and serology-based methods of diagnosis are also limited in this regard. While PCR based methods can be important in detecting co-infection with other pathogens

(Ben-Zvi et al., 2012), they cannot differentiate among the clinical malaria types, and they are also technically demanding and costly (Ansah et al., 2013). Serology-based methods of detection are limited in diagnosing onset of malaria; this is because detectable parasite specific antibody may only appear several days into the infection and will persist after treatment.

The most commonly used method of diagnosis in a district laboratory in sub-Saharan Africa is based on clinical signs and symptoms (Font et al., 2001, Aidoo, 2013). Resources and trained personnel are scarce in most areas where malaria is holoendemic. As a result, presumptive clinical diagnosis is predominant, and may result in the administration of anti-malarial drugs to individuals that do not need them (Olivar et al., 1991, Allen et al., 2013). The considerable symptom overlap between, for example, respiratory tract infection and malaria can lead to misdiagnosis and over-use of anti-malarial drugs (Redd et al., 1992, Aipit et al., 2014).

Efforts in the past to improve the specificity of presumptive clinical diagnosis have been limited (Smith et al., 1994). The WHO Integrated Management of Childhood Illnesses (IMCI) recommended that every child living in a high transmission malaria holoendemic region, where 5% of febrile children are parasitaemic, should be considered to be infected and treated for malaria (WHO, 1997). However, this is likely to produce a significant rate of malaria over-diagnosis.

In a bid to further understand differences in the plasma proteome of children presenting with different malaria symptoms, we observed some potential biomarkers that could discriminate among the malaria cases at onset and could be of importance in the disease prognosis.

The results in this chapter describe the plasma levels of HPx, sCD163 and clusterin in malaria syndromes. We show that the plasma HPx levels varies among

the malaria groups at acute onset. In addition, an sCD163 level also varies during the early stages of the infection and could be useful to monitor the onset of the recovery phase in response to treatment. Importantly, we have found that plasma Clusterin (CLU) level is a specific biomarker of CM and it's likely to have a protective role at the onset of the disease. The results here were obtained using the cohort described previously in table 3.1.

7.2 Plasma HPx and free-haem levels

7.2.1 Plasma HPx levels vary among malaria syndromes at onset and increases sensitively following treatment.

Plasma HPx levels among the disease groups are significantly different at onset (Fig. 7.1A-B). The plasma HPx level is statistically higher in the UM group than that of the severe malaria groups at onset (Fig 7.1A-B). Circulatory HPx levels among the severe forms of malaria are significantly different with distinct differences in median and mean values ($p<0.0001$, $F=10.08$) in spite of the closely related pathophysiology in some groups such as CM+SMA, CM and SMA (Fig 7.1A).

More importantly, the HPx levels in the malaria groups are different to that of the malaria negative DC group of mostly children presenting with fever of bacterial aetiology (Fig. 7.1A).

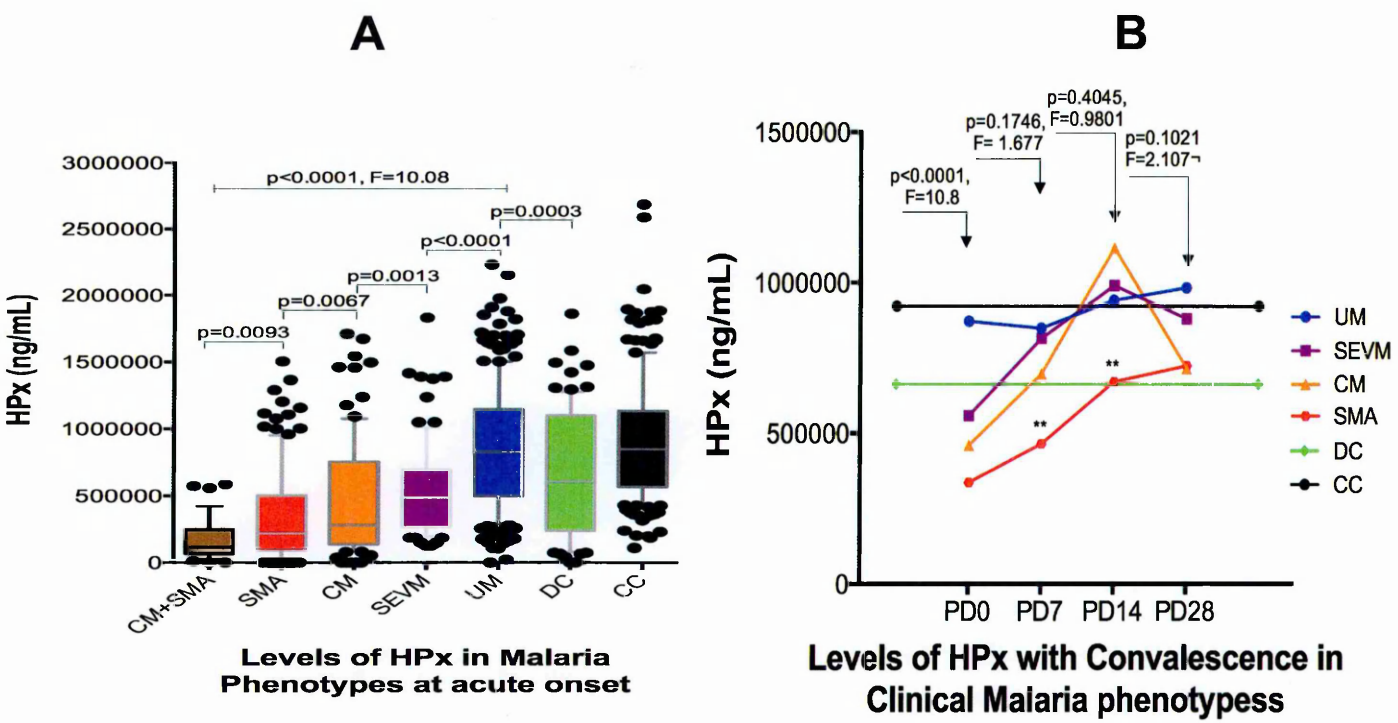
However, as found for other clinical biomarkers, there is some degree of overlap in levels but the level within each group has a distinct median and mean through convalescence (Fig. 7.1B).

The SMA group has a lower level of HPx at onset and all through convalescence to full recovery (Fig. 7.1B). Both the SEVM and CM groups show peak values at PD14 that are not statistically different to the level at recovery; PD28 (Fig. 7.1B).

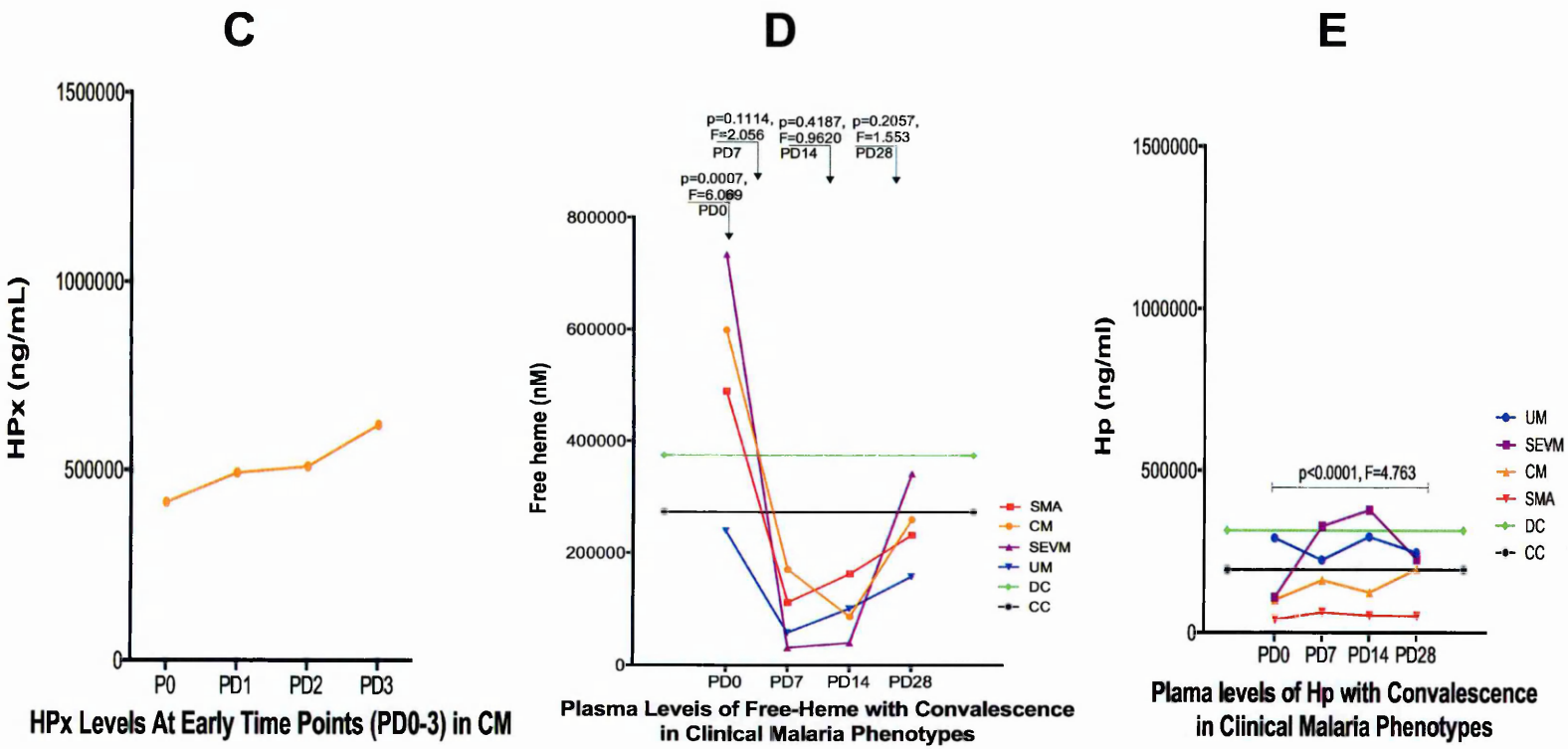
It is obvious that there is a gradual increase in HPx levels following drug treatment (Fig. 7.1B). Examination of the early time points (day 0-3) in the CM group shows that the plasma HPx level immediately begins to increase on commencement of treatment, indicating a quick response (Fig. 7.1C).

The levels of Hp at onset through convalescence to full recovery are unlike the levels of HPx (Fig. 7.1B and E). Levels of Hp in the malaria groups are low and stable through convalescence (Fig. 7.1E).

Figure 7.1 A) Levels of HPx at PD0; B) Levels of HPx with convalescence; C) Levels of HPx at early time points in the CM; D) Levels of Free haem with convalescence; D) Levels of Hp with convalescence



The graph in Fig.7.1A is shown as median and interquartile range with 10-90 percentile. The numbers in each group in Fig. 7.1A are based on the numbers at PD0 in the general cohort (Table 3.1). Samples common to all time points are used for Fig. 7.1B. Non-parametric ANOVA and Mann Whitney tests were used to analyze more than two groups and two groups of data respectively. Fig 7.1B is based on the mean values from each group.



C) HPx levels increase sensitively with treatment at early time points; day 0-3. The number of data point at each time point in Fig. 7.1C is 5 and their mean values are presented on the graph. This early time points data was only available for the CM group. Fig. 7.1D) shows the free haem dynamics in the disease groups. Data was based on full complement of samples in the general cohort (Table 3.1).

A non-parametric ANOVA was used to analyze data among groups. A $p<0.05$ was considered as being statistically significant. The F values measure the magnitude of the statistical level of significance as indicated by the p-value.

7.2.2 Plasma HPx levels correlate with parasite density and sCD163 level at onset

The plasma HPx level in the SMA group correlates positively with parasite density (PD) and negatively with sCD163 level at onset (Table 7.1a and d). There are negative correlations between HPx and sCD163 levels in both the UM and SEVM groups but not in the CM group (Table 7.1d). There is no correlation between Hp and HPx in the SMA and CM+SMA groups unlike the positive correlation in other malaria groups (Table 7.1c). None of the malaria groups show correlation between HPx and fHb (Table 7.1b).

7.2.3. There are significant differences in plasma free-Haem levels among malaria phenotypes at onset.

The levels of plasma free-haem in the malaria groups are significantly different at onset (Fig. 7.1 D). Levels fall drastically in the early convalescence phase between PD0 to PD7 (Fig. 7.1D). Levels at PD7 and PD14 are not statistically different in all the malaria groups (Fig. 7.1D). There is an increase in plasma free-haem from PD14 to recovery (PD28). The plasma free-haem levels at recovery in the malaria groups are not statistically different to one another (Fig. 7.1D).

The level of free-haem in the SEVM group at onset is higher than other groups but its plasma HPx level is not as low as might be expected (Fig. 7.1D and A_B). However, there are decreasing levels of free-haem along with increasing levels of HPx at the convalescence phase in all malaria groups (Fig. 7.1B and D). Both CM and SMA groups have lower plasma HPx levels that are not statistically different to other malaria groups at recovery (7.1B and D). While the UM group

shows a significant negative correlation (r=-0.3229; p=0.0063) between plasma HPx and free-haem levels, the severe malaria groups do not show any significant correlation. Free-haem levels show no correlation with either fHb or PD in all malaria groups.

Table 7.1 Correlation between HPx and a.) Parasite Density, PD; b.) fHb; c.) Hp; and d.) sCD163 at PD0

Malaria Syndromes	**a**. HPx and PD		**b**. HPx and fHb		**c**. HPx and Hp		**d**. HPx and sCD163	
	r	p	r	p	r	p	r	p
UM	-0.02178	ns	0.03258	ns	0.4874	< 0.0001	-0.1212	0.0140
SEVM	0.1024	ns	-0.06555	ns	0.3268	0.0066	-0.4930	0.0160
CM	-0.03812	ns	-0.1266	ns	0.4301	0.0001	-0.2083	ns
SMA	0.4106	< 0.0001	-0.03758	ns	0.06698	ns	-0.5942	0.005
CM+SMA	0.1492	ns	0.2074	ns	0.05086	ns	ND	ND

Non-parametric spearman correlation was used to compute the data. r = correlation coefficient; ns= not statistically significant; ND= not determined as sCD163 data was not available for CM+SMA group. This data is based on the general cohort table in Fig. 3.1, which contain the number of sample in each group.

7.3 Plasma levels of sCD163 in malaria groups

7.3.1 Plasma sCD163 levels in the UM group show changes not observed in other malaria groups

The plasma sCD163 level in the UM group increases significantly from PD0 to PD7; peaked at PD7 and thereafter decreases significantly to PD14 and then PD28. Level at PD28 is the same as that of CC group (Fig. 7.2A). The UM group has significantly higher plasma sCD163 level than other malaria groups at onset (Fig. 7.2E). The sCD163 levels in the SEVM group follow similar pattern as the UM group but levels at PD14 were not different to PD28 but normalized at PD14 to the same level as in the CC group (Fig. 7.2B).

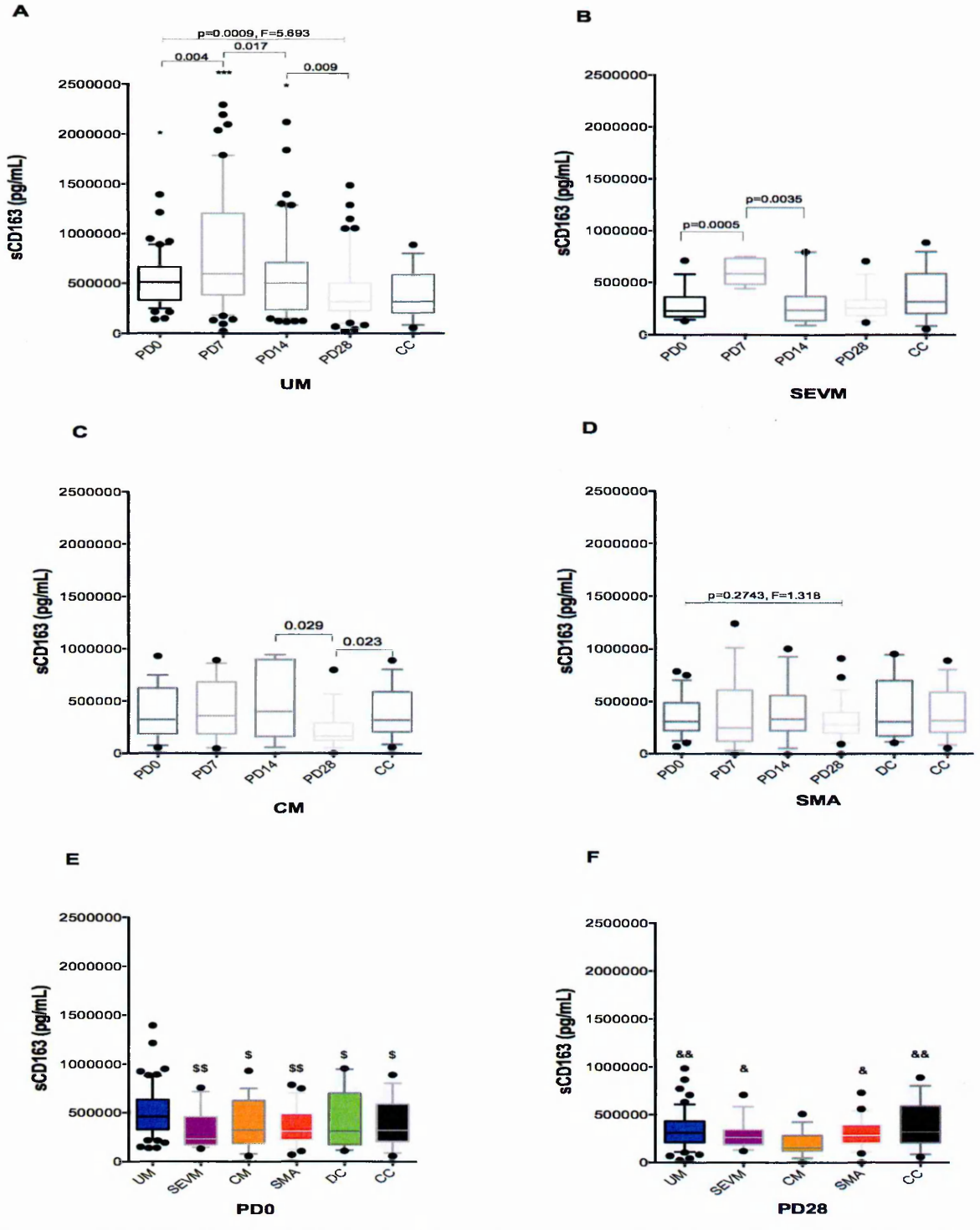
Unlike in both the UM and SEVM groups, plasma sCD163 levels in the SMA group are stable through convalescence (Fig. 7.2D)

Interestingly, the plasma sCD163 level at recovery in the CM group is significantly lower to levels in other disease groups and the CC (Fig. 7.2C and F). Aside from the reduced sCD163 levels in the CM at recovery, levels at both onset and recovery are the same in the entire severe malaria groups (Fig. 7.2E-F).

7.3.2 Correlation between sCD163, parasite density, fHb and Hp

Plasma Hp and sCD163 levels correlate negatively only in the DC group (Table 7.2b). No correlation between sCD163 and Hp is observed in the malaria groups (Table 7.2b). The plasma sCD163 and fHb levels show significantly negative correlation in the DC group and a significant positive correlation in the SMA group (Table 7.2a). There are no significant correlations between sCD163 and fHb levels in the rest of the malaria groups (Table. 7.2a). Plasma sCD163 and PD show significantly positive correlations in both the SMA and UM groups (Table 7.2c).

Figure 7.2 Plasma levels of sCD163 with convalescence in the A) UM; B) SEVM; C) CM; D) SMA; and in the different disease group at E) PD0 and F) PD28



Graphs are shown as median and interquartile range with 10-90 percentile. The data is based on the general cohort table in fig. 3.1. Non-parametric ANOVA and Mann Whitney tests were used to analyze the data. Fig 7.2 E and F are to allow comparison of levels at PD0 and PD28 within the different malaria groups. $p<0.05$ was considered as significant. *= Statistically different to CC (*=$p<0.05$; **=$p<0.01$; ***=$p<0.001$; ****=$p<0.0001$). $=Statistically different to UM ($=$p<0.05$; $$=$p<0.01$). &= Statistically different to CM (&=$p<0.05$; &&=$p<0.01$).

Table 7.1 Correlations between sCD163 and a) fHb; b) Hp; c) PD at PD0

Malaria Syndromes	**a**. sCD163 and fHb		**b**. sCD163 and Hp		**c**. sCD163 and PD	
	r	p	r	p	r	p
UM	0.217	ns	0.073	ns	0.240	0.032
SEVM	-0.261	ns	-0.251	ns	0.044	ns
CM	0.31	ns	-0.08	ns	0.164	ns
SMA	0.36	0.04	-0.03	ns	0.498	0.0035
DC	-0.39	0.04	-0.60	0.0045	ND	ND

Non-parametric spearman correlation was used to compute the data. r = correlation coefficient; ns= not statistically significant; PD = Parasite density; ND= not determined as PD is not available for the DC group. This data is based on the general cohort table in Fig. 3.1, which contains the number of samples in each group.

7.4 Plasma clusterin (CLU) levels in the malaria groups

Studies on CLU levels were performed on a separate paediatric cohort to those previously described. A total of 194 samples were used and consist of samples from each malaria disease group and those that died following CM attack (CM dead subjects) and including samples at both acute onset (PD0) and at recovery; PD28 (Table 7.3). The DC groups in this cohort consist of subjects with syndromes that closely mimic that of CM and include children with convulsion, mild anaemia and coma of other etiologic cause that include meningitis. A trained laboratory scientist not part of this study group confirmed the DC and the CC groups to be malaria parasites negative. The clinical characteristics of subject in the CLU cohort are shown in table 7.3 below. The plasma CLU level was assayed using a monoclonal anti-human CLU antibody.

7.4.1 Lower CLU level is associated with onset of CM

The plasma CLU level in the CM group is significantly lower than the levels in all other clinical groups at onset aside the levels in CM dead subjects (Fig. 7.3A). CM dead subjects have the least plasma CLU level, significantly lower than level in the CM group. CM dead subjects could not survive till day 28 (Fig. 7.3A).

Plasma CLU in the SMA group at onset shows a non-significant lower median to those of the UM, DC and CC groups (Fig. 7.3A). On recovery, the plasma CLU level in the CM and the rest of the malaria groups are not statistically different to the levels in the CC group (Figure 7.3B).

7.4.2 Plasma CLU level in the entire CM group increases with recovery

The circulatory CLU levels in the entire CM patients increase steadily from onset to recovery and appear not to be affected by individual variability in the

population (Fig. 7.3C). The mean level at recovery is significantly higher (p=0.0001) to levels at onset (Fig. 7.3C).

Plasma CLU levels in the SMA patients vary from onset to recovery; some decreases, others increases while some are stable (Fig. 7.3D). In the UM group, levels at onset are not statistically different to those at recovery. However, there is a slight increase from PD0 to PD28 in the majority of the patients (Fig. 7.3E).

7.4.3 Specificity and sensitivity of clusterin levels in discriminating among malaria syndromes

The area under the ROC curve termed as Area in the table below each ROC curve (Fig. 7.4A-F) is the measure of how well CLU level can distinguish between two disease groups; CM versus CC or CM versus SMA or UM groups.

Plasma CLU at acute onset (PD0) significantly discriminates CM from CC in approximately 91% of the subjects; Area 0.9087, P<0.0001 (Fig. 7.4A). The ROC analysis of levels at day 28 in the CM group does not show significant difference to the CC group (Fig. 7.4B). Plasma CLU levels significantly differentiate CM dead from healthy CC in 87% of the subjects considering the area under the curve; 0.8667, p<0.0001 (Fig. 7.4C). The ROC analyses further show that plasma CLU levels discriminates CM from DC, SMA and UM at acute onset (Fig. 7.4D-F).

7.4.4 Plasma CLU levels correlate differently with IL-6 and IL-10

Plasma CLU level shows significant negative correlation with the levels of IL-10, IL-6 and IL-8 only in the CM group at onset (Table 7.4). At recovery, both plasma CLU and IL-10 levels show significant positive correlation in the CM group and a significant negative correlation in the SMA group (Table 7.4).

The plasma IL-6 level correlates negatively with CLU level only in the SMA group at recovery (Table 7.4). There are no correlations between plasma CLU and these cytokines in the UM group (Table 7.4)

Table 7.3 Clusterin Cohort Table

Variables	Groups					
	SMA	**CM**	**UM**	**CM Dead**	**DC**	**CC**
PD0 (N)	30	27	18	7	12	30
PD28 (N)	19	20	17	N/A	N/A	N/A
Age (mean)	37	49	45	45	55	55
Sex (F/M) (N)	15/14	12/15	8/10	3/4	5/7	17/13
Parasite Density Mean with range (IQR)	87002[§] (11327-210275)	86559[§] (10240-191133)	64907 (3647-170348)	79648[§] (9759-191045)	0	0
PCV (%) median (IQR)	13 (5-21)	26 (17-40)	32 (16-42)	25 (18-37)	21 (15-23)	35 (27-40)

This table shows the number of subjects used for the clusterin studies. Male and female are represented in the cohort. The parasite density (PD) in the severe malaria groups are significantly higher than the UM. The SMA group has the lowest PCV value. Subjects that died of CM were designated as CM dead and were included in the study. N=Number, IQR- Interquartile Range, F=females, M=Males, N/A= Not Applicable, [§]statistically higher than UM ($P<0.05$).

Figure 7.3 Plasma Clusterin levels at A) PD0; B) PD28 and at onset and recovery in the C) CM; D) SMA and E) UM

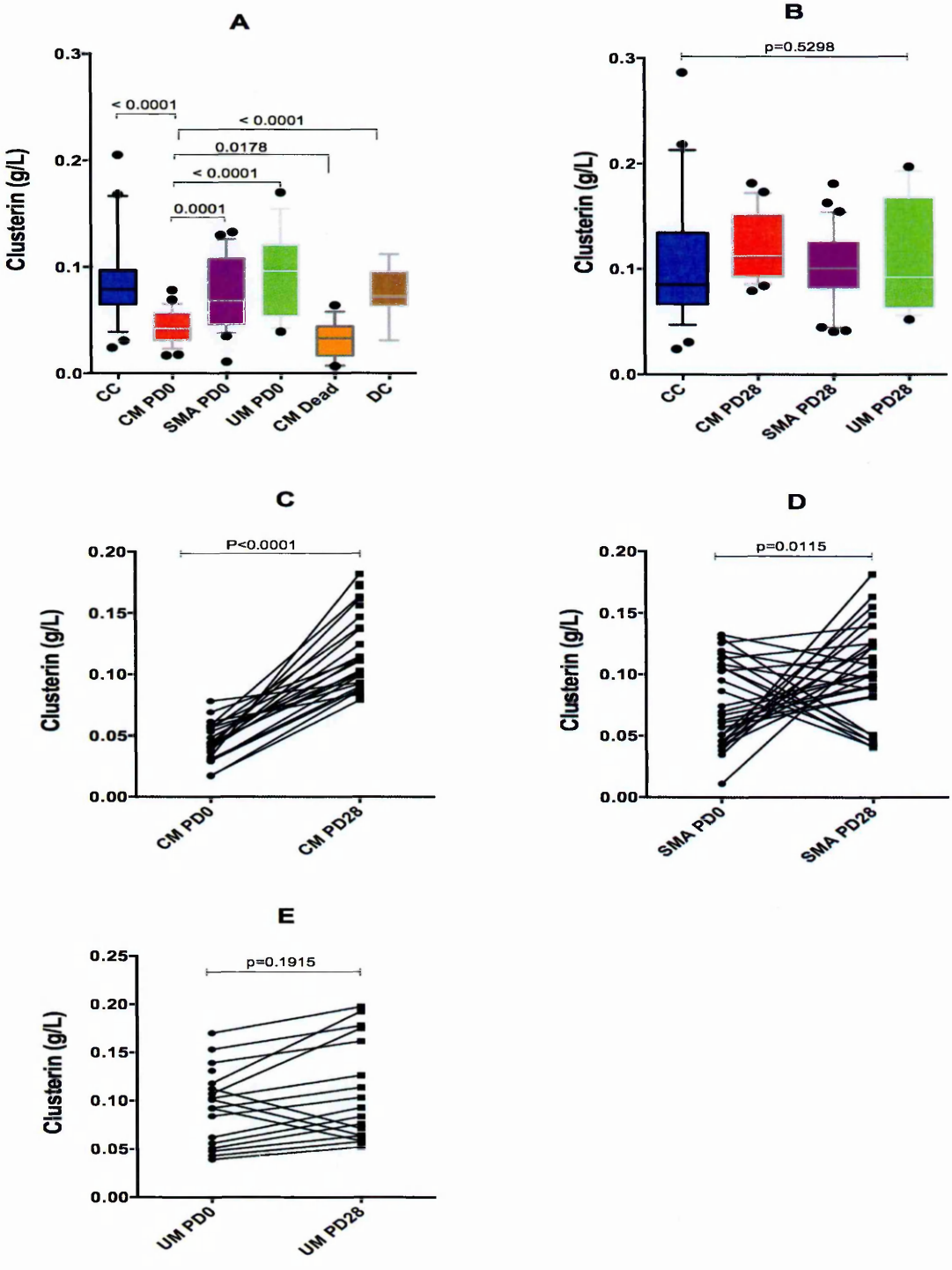
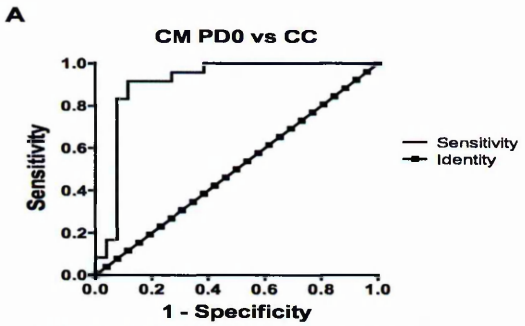


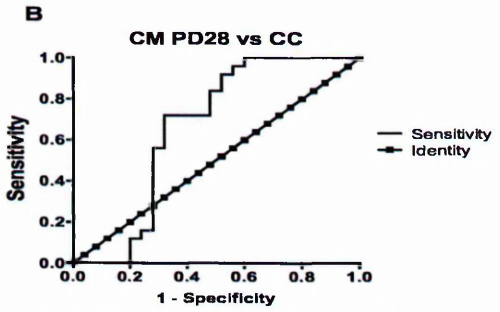
Fig. 7.3A-B) Data are presented as median interquartile range with 10-90 percentile. Non-parametric ANOVA and ANOVA with multiple comparison testing were used to analyze the data.

Fig. 7.3C-E) Plasma Clusterin levels in the CM, SMA and UM at both PD0 and 28. Data are presented as point graph from PD0 to PD28 and analyzed by paired non-parametric t-test. $p<0.05$ is considered as significant . The numbers of subjects in each group are as indicated in table 7.3.

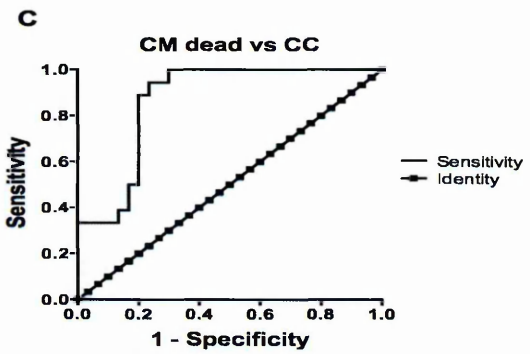
Figure 7.4 ROC curve analysis of plasma CLU levels



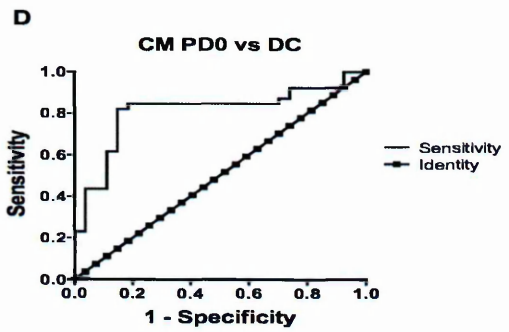
	Results
Area	0.9087
Std. Error	0.04825
95% confidence interval	0.8141 to 1.003
P value	< 0.0001



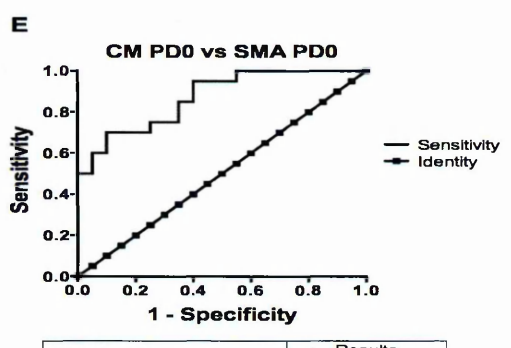
	Results
Area	0.6576
Std. Error	0.08470
95% confidence interval	0.4916 to 0.8236
P value	0.05604



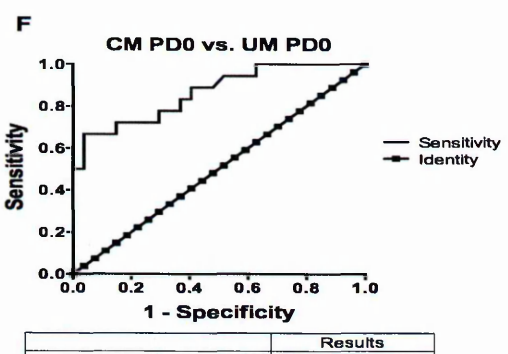
	Results
Area	0.8667
Std. Error	0.05219
95% confidence interval	0.7643 to 0.9690
P value	< 0.0001



	Results
Area	0.8101
Std. Error	0.05632
95% confidence interval	0.6997 to 0.9205
P value	< 0.0001



	Results
Area	0.8700
Std. Error	0.05412
95% confidence interval	0.7639 to 0.9761
P value	< 0.0001



	Results
Area	0.8632
Std. Error	0.05586
95% confidence interval	0.7537 to 0.9727
P value	< 0.0001

Plasma Clusterin level discriminate CM from other malaria syndromes and the DC in a ROC curve analysis. **(A)** ROC curve compares CM at PD0 to CC; **B)** CM at PD28 with CC; **C)** Dead CM subjects with CC; **D)** CM at PD0 with CC; **E)** CM at PD0 with SMA at PD0; F) CM at PD0 with UM at PD0. Under each curve is a statistical table showing the Area under the curve (Area), Standard error, 95% Confidence Interval (CI) and p-values. The area under curve quantifies the overall ability of the test to discriminate between two groups. For example, an area of 0.9087 or 90.87% as in the CM PD0 vs CC (Fig. 2A) means, the probability that a randomly selected CM patient will have lower clusterin level compared to the CC is 90.67% or 0.9067. Sensitivity indicates true positive rate while 1-specificity indicate false positive rate. p-value<0.01 was considered to be statistically significant.

Table 7.4 Correlations between CLU and cytokines levels at both onset and recovery

CLU	CM			SMA			UM		
	IL-10	IL-6	IL-8	IL-10	IL-6	IL-8	IL-10	IL-6	IL-8
Onset (PD0)	r=-0.5333 p=0.04	r=-0.6220 p=0.0101	r=-0.4989 p=0.0361	r=0.1091 p=ns	r=0.06364 p=ns	r=0.2545 P=ns	r=-0.6571 p=ns	r=0.02857 p=ns	r=0.1429 p=ns
Recovery (PD28)	p=0.6167 r=0.0429	r=0.3500 p=ns	r=0.3833 p=ns	r=-0.5593 p=0.0465	r=-0.7842 p=0.0044	r=0.06079 p=ns	r=0.7143 p=ns	r=0.3714 p=ns	r=0.3143 P=ns

Plasma CLU levels and that of IL-10, IL-6 and IL-8 at both PD0 and PD28 are analyzed by spearman correlation in subjects with CM, SMA and UM. $P<0.05$ is considered as statistically significant. r = the regression coefficient. Ns = not significant. The numbers of subjects in each disease groups are as shown in table 7.3.

7.5 Discussion

7.5.1 Plasma HPx levels significantly varies among the clinical malaria syndromes at acute onset

The management of malaria patients largely depends on the syndrome or clinical manifestation. Discriminating between the different clinical presentations of malaria at onset is important for appropriate therapy and to avoid unnecessary anti-malarial drug use.

The varying levels of plasma HPx in subjects with clinical malaria syndromes at acute onset in spite of related pathophysiology could be useful in assessing the severity of the disease. The rapid increase in plasma HPx level following appropriate treatment also makes it a valuable prognostic maker for effective malaria therapy.

More importantly, plasma HPx levels were able to discriminate between the UM and DC groups. This is important to discriminate between UM associated fever and fever of bacterial origin.

HPx is a better marker for diagnosis and prognosis in clinical malaria compared to Hp, particularly in a population with congenitally low Hp levels. The congenitally low level of Hp hampers its usage as a biomarker in haemolytic inflammatory disease.

The plasma HPx is recycled and never completely depleted unlike plasma Hp. HPx has a longer half-life of about 7 days in circulation, allowing for more precise measurement compared to the few minutes half-life of Hp (Langlois and Delanghe, 1996, Delanghe and Langlois, 2001, Echeverry et al., 2016). Furthermore, the HPx level is not influenced by extravascular haemolysis unlike other markers of haemolysis and its level is not influenced by ahaptoglobinaemia/hypohaptoglobinaemia in healthy subjects (Langlois and

Delanghe, 1996, Delanghe and Langlois, 2001). All of these make HPx a better marker in the diseased subjects. This finding is important in African populations with high frequency of a/hypohaptoglobinaemia as observed in this study and in line with previous reports (Delanghe and Langlois, 2001, Constans et al., 1981, Allison et al., 1958, Carter and Worwood, 2007).

The absence of correlation between HPx and free-haem in the severe malaria disease groups could be due to the proposed endothelial sequestration of haem (Balla et al., 1993, Balla et al., 2005, Camus et al., 2015).

An inverse correlation between HPx and free-haem that only exists in the UM group and the direct correlation between HPx and Hp in the UM, SEVM and CM groups that are absent in the SMA group is due to the naturally low Hp and suboptimal HPx level in the SMA group. This lower than normal levels of both Hp and HPx predisposes to SMA.

The significant positive correlation between HPx and PD in the SMA is an indication that they both increase together. We propose that the increase in PD triggers HPx expression via the release of free-haem by parasite action on the erythrocytes. However, the suboptimal level of HPx in the SMA group exacerbates the pathological process. The increasing PD also seems to trigger the expression of CD163 receptors, which could accounts for the significant positive correlation between both of them in the UM and SMA groups. The CD163 receptors are also likely to be involved in the direct endocytosis of both free-haem and fHb particularly in the event of low Hp level as in the SMA group. Both the uptake of free-haem and fHb by CD163 receptors leads to subsequent shedding of the receptor as sCD163. The sCD163 released is also involved in the binding of fHb (Madsen et al., 2004), which could again account for the observed positive correlation between fHb and sCD163 in the SMA group. The correlation between sCD163, fHb and Hp in the DC group reveals the supposed homeostatic

mechanism and the deviation from this as observed in malaria contributes to disease severity.

The increasing level of free-haem at the recovery phase as HPx levels returns to normal could be due to erythrocyte turnover rather than a consequence of the pathology.

A more precise way of malaria diagnosis and monitoring response to treatment is to measure the levels of HPx in tandem with free-haem levels. This approach could also be used in parallel with MRDTs, microscopy and clinical observation in a district laboratory in order to properly discriminate among the clinical malaria syndromes at acute onset.

7.5.2 Plasma sCD163 as a marker for malaria prognosis

Plasma sCD163 level is a useful measure to monitor macrophage activation in inflammatory conditions (Dige et al., 2014, Kjaergaard et al., 2014). The expression of CD163 on the surface of monocytes is accompanied by increasing sCD163 (Cunnington et al., 2012).

The high level of sCD163 in the UM group at onset and the increase in levels from PD0 to PD7 in both the UM and the SEVM groups during treatment might likely indicate an onset of the recovery phase and appropriate response to the treatment. Treatment appears to reactivate the action of the macrophages during the recovery phase through an as yet unknown mechanism. However, the immunomodulatory effects of anti-malarial drugs have been reported (Zhao et al., 2012). The recruitment of monocytes and macrophage activation are essential for the clearance of malaria parasites (Chua et al., 2013a), which probably include those parasites that are killed by drug action.

Furthermore, sCD163 is known to have anti-inflammatory and cytokine-like functions and was reported to be associated with the healing phase of

inflammatory process (Zwadlo et al., 1987, Van Gorp et al., 2010, Sulahian et al., 2004, Moestrup and Moller, 2004, Frings et al., 2002). Therefore the stage at which it is expressed at a higher level could mark the onset of an anti-inflammatory response and the recovery process rather than disease progression. Therefore, day 7 when the sCD163 level peaked is the onset of the recovery phase in the UM and the SEVM groups unlike PD14 in the CM group following drug treatment. The subsequent decrease of sCD163 to normal level in the UM group post PD7 is a sign of disease resolution and recovery. This reduced level post PD7 after the initial increase may be considered as a marker of effective malaria therapy.

Unlike the pattern in the other malaria groups, the lower level of sCD163 in the CM group at recovery could be a predisposing factor to CM. This low level in CM is neither due to fHb or Hp level. As proposed earlier in chapter 4, we considered the level at full recovery as comparable to the levels prior to the disease. The stable levels of sCD163 at onset and through convalescence to recovery in the SMA groups indicate poor macrophage activation and it is likely to be a predisposing factor for the onset of SMA.

An elevated level of sCD163 is associated with protection from malaria and delay of disease onset (Perdijk et al., 2013, Kusi et al., 2008). Therefore, the more drastic CM episode as reported (Pasvol, 2005, Idro et al., 2010) could be tightly linked to the suboptimal sCD163 level.

7.5.3 Plasma CLU level is a specific marker for CM at onset.

We show that low plasma CLU is tightly associated with the pathophysiology of CM at acute onset and discriminates CM subjects from other malaria syndromes and those with closely related syndromes such as convulsion, anemia and coma: the DC. We propose that high levels of plasma CLU are needed to enhance the recovery process in the CM group. Furthermore, the

depleted plasma CLU level in the CM group at onset discriminates this group from other severe malaria groups. Its increasing level with patient recovery is indicative of a better prognosis in the CM group.

The protective role of plasma CLU at the onset of CM may be linked to its functions that are tightly related to the pathophysiology of CM. One major CM pathophysiology is thought to be due to mechanical obstruction of the cerebral microvasculature by infected red blood cells (RBC) and neurological dysfunction due to blood-brain barrier permeability that allows parasite product access to the brain (Polimeni and Prato, 2014). Hence, CM is characterized by neurological dysfunction and encephalopathy-like syndrome such as psychosis, headache, seizure and coma (Newton and Warrell, 1998, Gitau and Newton, 2005, Polimeni and Prato, 2014).

Plasma CLU has been reported to accumulate at fluid-tissue boundaries where it carries out a cyto-protective role. It could act either as a surface bound or diffusible form to protect membranes (Wilson and Easterbrook-Smith, 2000). As a result of this, its biologic function at the blood brain barrier and regions of membrane attack due to parasitized RBC cytoadherence and sequestration is more likely.

Plasma CLU is known to prevent neurodegenerative disorder, a hallmark of CM. CLU's primary source of secretion is neural cells (Nuutinen et al., 2009, Sokolowski and Mandell, 2011), and it is involved in repair and replacement of nervous system cells that have lost the ability to regenerate (Jones and Jomary, 2002, Nuutinen et al., 2009, Sokolowski and Mandell, 2011).

The primary function of CLU is to promote cell clustering (Shafie et al., 2014), which could be important in the recruitment of cells such as phagocytic cells to sites of parasite sequestration. CLU itself is known to have phagocytes like function in cells exposed to cellular debris (Bartl et al., 2001). It also functions as a

chaperone, plays a role in maintaining stressed protein in a stable folded state, serves as an extracellular heat shock protein and protects cells from heat and other stressful stimuli (Wilson and Easterbrook-Smith, 2000, Michel et al., 1997, Luo et al., 2005).

Plasma CLU is reported to function as an anti-oxidant and is likely to prevent cellular apoptosis and enhance viability (Wang et al., 2014b, Luo et al., 2014, Zhang et al., 2014). Its antioxidant role is closely linked to the prevention of cellular senescence caused by cellular stress and induced DNA damage (Luo et al., 2014).

Conditions of stress such as hypoxia and cellular damage that are typical of CM (Clark et al., 2006) are known to induce the expression of CLU to prevent cellular apoptosis (Jones and Jomary, 2002, Trougakos and Gonos, 2009, Reddy et al., 1996, Wang et al., 2014b, Luo et al., 2005) through its interaction with the pro-apoptosis protein, Bax (Zhang et al., 2005, Kim et al., 2012b). *CLU* gene knock out in human cells causes a significant reduction of cellular growth and a higher rate of apoptosis (Trougakos et al., 2004).

CLU also plays a role in the prevention of inflammation: mice deficient in CLU are reported to have severe inflammation (McLaughlin et al., 2000). Complement deposition is known to be involved in clearance of infected RBC in CM (Eisenhut, 2015, Poh et al., 2014) and plasma CLU is known to function as a complement inhibitor (Nuutinen et al., 2009, Sokolowski and Mandell, 2011, Jenne et al., 1991, Jenne and Tschopp, 1992).

Interestingly, recent studies have implicated Matrix Metalloproteinases (MMPs) in the pathology of CM. These MMPs play several roles in the development of CM, for example they bind to a large repertoire of substrates and induce an imbalance between homeostatic molecules, increase pro-inflammatory responses, and enhance blood brain barrier permeability by disrupting the

endothelial junction (Polimeni and Prato, 2014). Several antimalarial drugs, such as chloroquine and artemisinin and its derivatives are known to either reduce expression or circulatory levels of several MMPs (Lesiak et al., 2010, Buommino et al., 2009, Wartenberg et al., 2003, Polimeni and Prato, 2014). At present, broad spectrum and/or specific MMP inhibitors are being sought as an adjunct therapy in CM (Polimeni and Prato, 2014). CLU is known to act as an MMP inhibitor. It binds very strongly to MMP-9, MMP-2, MMP-3 and MMP-7 and inhibits their enzymatic activity (Jeong et al., 2012).

The low plasma level of CLU correlates with the high IL-6 level at onset while the high CLU level at recovery correlates with IL-10 level. We therefore propose that the late expression of IL-10 and continuous expression of both IL-6 and IL-8 could exacerbate the pathology of CM by decreasing the level of CLU. The correlation between IL-6, IL-10 and plasma CLU levels in the CM group is opposite to that in the SMA group and both appear to be regulated over time. This result is consistent with reports that the roles of cytokines in malaria are tightly regulated (Burte et al., 2013, Xu et al., 2013, Noone et al., 2013, Robinson et al., 2009).

In conclusion, we show that HPx levels discriminate between all the clinical malarial disease groups and between clinical malaria syndromes and the DC group at onset. Changes in the levels of HPx and free-haem through convalescence may be used as a prognostic marker during appropriate treatment. Plasma sCD163 levels can also be considered as a prognostic marker in malaria as well as the marker for effective malaria therapy, particularly in the UM group. We suggest that low and stable level of sCD163 could predispose to CM and SMA respectively. Importantly, we show that low plasma CLU level is a specific marker for CM and therefore suggest that parenteral administration of CLU may enhance recovery from CM and prevent its associated neurological sequelae such as

severe neurological injury (McGuire et al., 1994), and epilepsy (Christensen and Eslick, 2015, Serghides et al., 2014).

Finally, we show that insight into malaria disease progression and recovery can be achieved by monitoring underlying differences that are associated with the pathophysiology of UM in comparison to those of severe malaria syndromes.

CHAPTER EIGHT

8.0 FINAL DISCUSSION

Malaria illness exhibits different clinical syndromes at presentation. While some develop just UM, others develop more severe forms such as SMA, CM, respiratory distress and hypoglycemia. In this study we showed the likely underlying pathophysiology and genetic factors that are associated with the acute or insidious onset of SMA.

As earlier mentioned in the Introduction, several mechanisms underlying the pathophysiology of SMA have been suggested. Though there are controversies regarding the appropriate models to test for these mechanisms (Craig et al., 2012), they appear to be linked to the pathology of severe malaria in general. However, there is no distinction as to which of the mechanism(s) is (are) commonly associated with a given severe malaria syndrome and whether these mechanisms are dependent on individual host factors or differences.

Most often than not, general mechanisms underlying the pathophysiology of severe malaria are presented without a drill down to specifically understand the pathophysiology of a given malaria syndrome such as SMA. This generalization could lead to wrong assumptions. For example, malaria associated intravascular haemolysis could be linked to low plasma Hp level but it is not right to assume that low plasma Hp level is a consequence of intravascular haemolysis.

Lack of properly stratified and clinically defined syndrome-specific based study could be linked to some failure or poor assessment of adjunct therapy in terms of benefit or harm (Cunnington et al., 2013). The different responses to malaria infection that manifested as syndromes could be the result of certain individual or host factors. Hence, stratification along such lines could be important.

In this study, samples are collected based on specific malaria syndromes.

In addition, we stratified samples based on Hp isoforms; a protein that is tightly linked to inflammatory and haemolytic disease such as malaria. This is because Hp is one of the important proteins expressed early in the pathophysiology of malaria that could set the stage or regulate other downstream homeostatic processes. Apart from its immunomodulatory functions as previously mentioned in the Introduction, it appears to set the stage for other proteins that might be implicated midstream and downstream in the infection stages such as markers of oxidative stress, CD163, HO-1, HPx, bilirubin, ferritin and cellular signals.

Importantly, we hypothesized that insight into acute onset and malaria disease progression and/or recovery can be achieved by monitoring the underlying pathophysiology of UM in comparison with those of other severe malaria syndromes at acute onset and through convalescence to recovery.

8.1 Likely mechanism(s) that could restricts infection to the UM.

Understanding the pathophysiology that could restrict malaria infection to the UM is important in order to understand the anomalies that are associated with the onset of the severe forms.

Aside from the genetic differences, these studies have shown that early response of Hp, HPx and sCD163 is very important and could restrict the infection to UM. The parallel increase in plasma levels of Hp, HPx and sCD163 and an effective homeostatic process between both Hp/fHb and HPx/free-haem could protect from severe forms of malaria. This is because low levels of both HPx and Hp could cause impairment in the scavenger-receptor pathways as well as other downstream homeostasis processes that are important towards recovery. The level of sCD163 could also be important in limiting disease progression but more effectively in the presence of Hp and HPx. This is because the protective role of sCD163 is enhanced and supported by both Hp and HPx. The null or low level of

plasma Hp affects the capacity of HPx to scavenge the free-haem overload. This becomes more complicated in individuals with suboptimal levels of both sCD163 and HPx as determined by ELISAs.

Furthermore, enhanced and early clearance of both fHb and free haem by scavenger-receptor mediated endocytosis has both anti-inflammatory and anti-oxidant effects as a result of the downstream by-products of the process such as sCD163, bilirubin, Fe^{2+} and CO. The sCD163 has anti-inflammatory roles that could restrict the infection; the Fe^{2+} is important in red cell iron turnover and erythropoiesis while the Fe^{2+}/ferritin confers cytoprotection (Otero et al., 2009, Lanceta et al., 2015). Interaction between sCD163 and IgG protects against the oxidative effect of Hb as recently suggested (Subramanian et al., 2013).

Finally, low parasite biomass or density could restrict the infection to UM rather than the severe forms except when there are other underlying conditions such as genetic factors and low or dysfunctional Hp and HPx level. The parasite density and pfHRP in this cohort are higher in the severe malaria groups than the UM.

8.2 Low plasma levels of Hp, HPx, CLU and sCD163 predisposes to severe malaria

Pathological conditions and/or genetic factor as with plasma Hp levels could cause low levels of plasma proteins such as HPx, CLU, sCD163 and Hp. The varying levels of Hp, sCD163, HPx and CLU are the common denominator among the entire malaria syndromes in this study.

The level of HPx and Hp in the SMA are suboptimal and it's likely to be responsible for the absence of correlation between HPx, free-haem, Hp and fHb in the SMA group. We have observed that intravascular haemolysis in the SMA group is not higher than in UM and other severe forms of malaria. However,

clearance of fHb seems to be impaired by low/null Hp and suboptimal HPx level in the SMA group. Results from the previous chapters have shown that this low level of Hp through convalescence and in the absence of higher haemolysis in the SMA group as compared to other malaria groups are likely to be genetic. The lower level of Hb in the SMA is not attributable to higher intravascular haemolysis but could be rather due to an enhanced clearance of both infected and uninfected erythrocytes by the spleen as previously mentioned in chapter 4. However, the reason behind this enhanced erythrocyte clearance in the SMA group compared to other disease groups is as yet not clear but previous reports had suggested that it could be linked to certain types of complements proteins as mentioned previously in the Introduction.

HPx is known to be recycled during haem metabolism and it's not expected to be depleted. Though high PD is a contributory factor to the development of severe forms of malaria, it appears not to be responsible for the suboptimal levels of HPx in both the CM and the SMA group. For example, the PD in the CM and SMA are not different to that of the SEVM group. Also, both CM and SMA have lower free-haem levels compared to the SEVM group yet their HPx levels are lower compared to levels in the SEVM group at PD28 (Fig. 7.1B and D). The lower level of HPx with convalescence in the SMA group does not appear to be associated with age. Therefore, the suboptimal HPx level in the SMA and CM groups at PD28 could constitute a risk factor rather than being a consequence of haem overload.

The expression of both Hp and HPx upstream of the infection process is determinant factor as to how the infection progress. Impairment of both Hp and HPx leads to accumulation of fHb; free haem; tissue damage; organ failure (Ferreira et al., 2008) and impaired sCD163 and HO-1 functions, which are tightly linked (Cunnington et al., 2004, Cunnington et al., 2012).

Furthermore, the stable level of sCD163 with convalescence in the SMA and the lower than normal level of sCD163 associated with the CM at PD28 constitute poor prognosis in both the SMA and the CM respectively,

The low plasma CLU level uniquely associated with the CM at acute onset not observed in other malaria syndromes might likely explain the development of CM in some subjects instead of SMA.

8.3 Haptoglobin phenotypes modulate response to malarial infection

The differences in the pathophysiology of severe malaria syndromes are linked to differences in Hp phenotypes and could be accountable for some contradictory reports in the studied subjects.

In this study, aside from clinically defined syndromes of malaria, we further sub-classified each syndrome into the 3 sub-categories based on the major Hp phenotypes. Both the 'slow and fast' Hp sub-phenotypes as previously described (Connell et al., 1966, Koch et al., 2003), were not considered, as the entire population have the same sub-phenotype of Hp.

Hp phenotypes have been reported to be associated with several non-infectious and infectious diseases including malaria. But it was not known if malaria associated pathophysiology are dependent on Hp phenotypes.

Plasma level of Hp and HPx tend to decrease with increase in PD in individuals with the Hp1 allele but increase with increase in PD in the Hp2 allele. Also, the low level of Hp in the SMA is more pronounced in the Hp1-1 phenotype. These are important in determining the pathological equation of the disease. The duration from the onset of the disease to recovery appears to be associated with Hp phenotypes; inflammation as indicated by CRP levels is easily resolved in SMA subjects with Hp1-1 and Hp2-1 but not Hp2-2 and subjects with Hp2-2 but without SMA and vice versa. Inflammation, oxidative stress and cytokines imbalance in

clinical malaria syndromes are divided along the lines of Hp phenotypes and are unlikely to be entirely involved at the same time within a given malaria pathophysiology.

Hp phenotypes appear to modulate the plasma levels of markers of intravascular haemolysis, oxidative stress, macrophage-associated chemokines and both pro/anti-inflammatory cytokines in clinical malaria. The levels of these plasma proteins are dependent on both the malaria and Hp phenotypes and are likely to explain the differences in malaria pathophysiology and disease severity.

Individuals with different Hp phenotype and presenting with the same malaria syndrome elicit different responses and vice varsa. Some likely physiological pathway to disease progression from mild to severe malaria or onset of severe syndromes within a given Hp phenotype could be deduced from the difference between the mild malaria to severe malaria syndromes as highlighted below.

8.3.1 Comparing UM to severe forms in Hp1-1 subjects

The depleted circulatory levels of Hp, IL-6 and higher circulatory levels of LDH, IL-8 and MIP-β (CCL4) in the SEVM subjects compared to the UM subjects are the major pathophysiology difference between uncomplicated malaria and respiratory distress in subjects with Hp1-1 phenotype. Elevated plasma CCL4 was reported in severe malaria (Perkins et al., 2011) while another report indicated that the level is also elevated in mild malaria as well (Ochiel et al., 2005). However, we observed that the level of CCL4 in malaria seems to vary with both Hp and malaria phenotypes rather than being restricted to either the UM or the severe forms. While the plasma CCL4 is high in the SEVM group with Hp1-1 and 2-2, its levels is lower in the SEVM subjects with Hp2-1 as in the mild or uncomplicated malaria.

Depleted circulatory plasma levels of Hp, stable MCP-1, higher LDH, early and

stable IL-8, high and stable level of fHb and low and stable level of sCD163 are found in the CM compared to the UM in subjects with Hp1-1 phenotypes. Several reports have associated high IL-8 with disease severity (Lyke et al., 2004, Bostrom et al., 2012, Mahanta et al., 2015), but it rather seems to be tightly linked to Hp phenotypes.

Null/low and stable levels of Hp, depleted level of CLU and lower IL10:IL-6 at onset in the SMA differentiates it to UM in subjects with Hp1-1. The imbalance between pro-inflammatory and anti-inflammatory cytokines through excessive production of IL-6 and IL-10 is more associated with Hp1 allele in the SMA group. The UM group have lower plasma Hp, SOD-1, IFN-γ and higher plasma LDH, CRP, IL-6, IL-10, and MCP-1 compared to the CC in subjects with Hp1-1.

8.3.2 Comparing UM to severe forms in Hp2-1 subjects

- Higher levels of LDH and low IL-10:IL-6 ratio differentiate SEVM from the UM in Hp2-1 subjects. However, the UM2-1 has higher CRP compared to the SEVM2-1.
- Depleted Hp, CLU, higher LDH and continuous expression of both IL-6 and IL-10 (low IL-10:IL-6) at onset differentiate CM from UM in Hp2-1 subjects.
- The SMA subjects with Hp2-1 have a very high level of SOD-1, G-CSF and low IL-10:IL-6 ratio at acute onset compared to UM subject with the same phenotype.
- Lower levels of IL-6, IL-10, and higher plasma IL-10:IL-6 and IFN-γ differentiate UM from CC in subjects with Hp2-1 phenotype.
- High plasma level of CLU and lower plasma levels of MCP-1, IL-6, and IL-10 in the UM differentiate them from the CC.

8.3.3 Comparing UM to severe forms in Hp2-2 subjects

- ❖ Lower Hp, IL-10:IL-6, IL-17 and higher levels of LDH, CRP and MIP-1B are associated with SEVM at onset compared to the UM in Hp2-2 subjects.
- ❖ Lower Hp, CLU, IL-10:IL-6 and higher LDH and CRP are associated with CM at onset compared to the UM in subjects with Hp2-2.
- ❖ Lower Hp, SOD-1 and CLU at acute onset and high IL-8 and GM-CSF at recovery differentiate SMA from the UM in subjects with Hp2-2.

Importantly, while the plasma proteins in the CC group do not show any major difference with Hp phenotypes that of the DC does. High plasma level of IL-17 and GM-CSF are associated with DC2-2 and high plasma level of both G-CSF and Hp are associated DC2-1 group while high LDH level is a hallmark of DC1-1 group.

Oxidative stress and cytokine imbalance appear not to play a role in the pathology of SMA in Hp2-2 patients unlike SMA in patients with Hp1 allele as was previously discussed in chapter 5.

8.4 Differentiating SMA from other severe haemolytic anaemia

We observed that the pathophysiology of severe anaemia in malaria is different to that of parasite negative severe anaemia of other aetiology (DC). While the low level of Hp in the DC is a consequence of haemolysis that of SMA is majorly congenital and could be a risk factor for acute onset of SMA. Also, there is a likely receptor-mediated uptake of fHb by CD163 in the DC while the process seems impaired in the SMA group. While low Hb level in the DC could be linked to intense intravascular haemolysis; that of SMA could be due to enhanced clearance of both infected and uninfected erythrocyte by the spleen.

8.5 Host genetic factors and severe malaria

Host genetic factors can confer susceptibility or protection to severe malaria. As earlier mentioned in Chapter 6, sequence variants at both distal and proximal upstream regions of the translational start site on *Hp* gene could confer susceptibility or protection to severe forms of malaria. Some of these variants determine the progress of the disease more than any anomalies in the pathological process. For example, the low plasma Hp level predisposes to SMA but the presence of the g.-49A/C genotype for the variant, g.-49A>C, which is associated with the reduced level of Hp protects from SMA. The g.-49A>C variant is linked to the contradictory report (Cox et al., 2007) regarding the plasma Hp levels and susceptibility or protection to malaria. The g.-1203 is also associated with severe malarial anaemia and plasma Hp levels.

We observed that several deletions at the distal upstream region of the Hp gene confer significant protection to SMA. Genotypes from variants at both the proximal and distal upstream regions of Hp gene show significant association with the various malaria syndromes.

Genotypes that are associated with Hp levels determine the susceptibility rate of Hp phenotypes to SMA. Congenitally low/null plasma Hp level as reported in this study is a predictor of whether or not a child with uncomplicated malaria will go on to developing an SMA episode in the clinic.

We have shown that the pathology of malaria, particularly the severe forms are tightly linked to *Hp* gene sequence variants despite indications that *it* could be equally important in other diseases. Hp genotypes show different frequencies with geographical region and ethnic groups. It therefore appears that these differences in Hp genotype with geographic location could be responsible for the contradictory report on malaria pathophysiology in different regions.

8.6 Potential biomarkers of clinical malaria syndromes

Biomarker discovery is very important in order to enhance quick and accurate diagnosis and prognosis. A major problem in biomarker discovery, particularly in severe malaria is due to overlapping pathophysiology manifested as syndromes.

Plasma CLU, a protein that is rarely reported in malaria could have application as a biomarker of CM. CLU levels in CM subjects are uniformly low at acute onset independent of the individual Hp phenotypes unlike in the SMA, UM and SEVM groups. Low levels of CLU appear to be linked to onset of CM.

The steady rise in plasma HPx levels in clinical malaria as earlier stated in Chapter 7 could have an application as a prognostic biomarker in malaria in predicting response to treatment. Changes in the levels of sCD163 at early time points during treatment could mark the onset of recovery.

8.7 Concluding remarks

This study was able to address the research questions as mentioned in Chapter 1. We show that intravascular haemolysis in the SMA is not higher than in other malaria syndromes and low/null Hp level is likely to be a risk factor for malaria and its severe forms rather than being a consequence of haemolysis. Furthermore, previously unknown variants at the proximal and distal upstream region of *Hp* gene were shown to have association with plasma Hp levels and/or disease.

The pathophysiology and response to malaria infection is modulated by Hp phenotypes. Individuals presenting with the same malaria phenotypes could mount different responses that are based on their Hp phenotypes. Therefore, difference in Hp phenotypes could explain some of the contradictory reports on the pathophysiology of malaria.

Hp is one of the important proteins elicited upstream of the infection stage in the pathology of malaria and appears to indirectly regulate the role of other midstream and downstream proteins.

This study further reveals the association between low plasma CLU level and CM at acute onset and the potential of HPx in predicting response to treatment.

The findings described here are particularly important in district clinics in the sub-Saharan region of Africa where malaria is holoendemic and both trained physicians and clinical scientists are largely absent.

8.8 Future Work

- We hypothesized that some kind of interaction at the distal and proximal upstream region of the Hp gene (~3.4kb) are involved in susceptibility to severe malaria, which needs to be investigated in a mechanistic study. There is a need to further understand the expression of Hp gene in terms of studying the transcription factors and some likely proteins that could interact with the proximal and the distal promoter region of the gene.
- Since Hp appears to modulate response to malaria infection, there is need to further study the immunomodulatory functions of the different Hp phenotypes in malaria. The association between Hp phenotypes and cytokines at acute onset of malaria needs to be studied in all the clinical presentations of malaria. Plasma cytokine levels, inflammation and oxidative stress appear to be divided along the lines of Hp phenotypes and are likely to be important for protection or susceptibility to severe forms of malaria.
- A mechanistic study is required to further investigate the role of plasma CLU in malaria particularly CM. Further study is required to test for the

biologic functions of CLU in line with the proposed mechanisms underlying the pathology of CM as proposed in this study. The therapeutic value of CLU in malaria also needs further investigation.

- There is need to investigate the possible glycosylation pattern and its role in malaria in both the β and α-chains of Hp. In Fig. 3.1C-D, four spots labeled as 3, 4, 5 and 6 with slightly different PI and molecular weight were identified as Hp (Fig. 3.2C) and two spots, 9 and 10 were identified as Hp α-2 chains (Fig. 3.2D). We observed that spot 3 (Fig. 3.2C) is very faint in the entire clinical groups while spot 6 appears to be more expressed in the UM than the CC. Both spots 9 and 10 of the α-2 chain are depleted in the SMA group but only spot 10 is depleted in the CM group (Fig. 3.2D). All these spots were identified as Hp by mass spectrometry (Table 3.2). We proposed that glycosylation is more likely to explain the slight differences in the pI and molecular weight of the Hp spots and their different levels of expression could likely indicate different roles in malaria. A study in Non-small Cell Lung Cancer patient had reported similar spots of α-2 chain with a isoelectric point (pI) of about 5.95 and 5.65, which gave a strong positive signal with fluorescein labeled WGA (Wheat Gel Agglutinin) and could indicate possible glycosylation with sialic acid and/or N-Acetylglucosamine (Everaert et al., 1998a). Hp is widely reported in many diseases and its functions are likely to be affected by their glycosylation pattern. Sialylated glycoprotein has been reported to mediate binding between Hp and mature B-cell expressing CD22 (Powell et al., 1993, Hanasaki et al., 1995).

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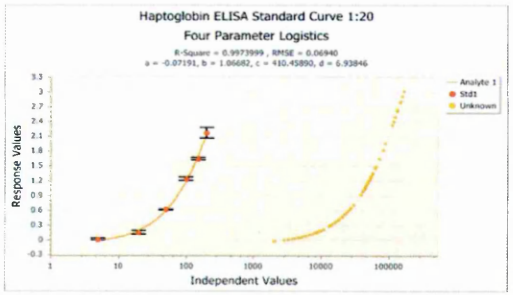
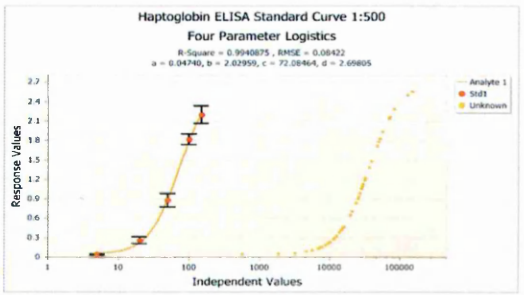
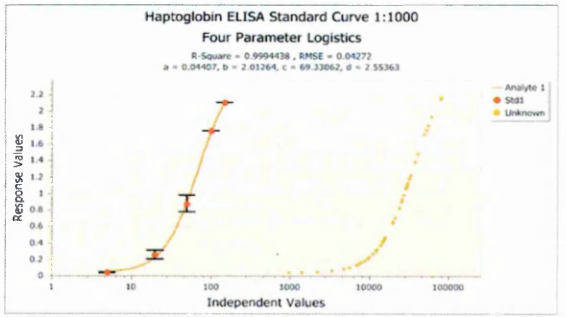
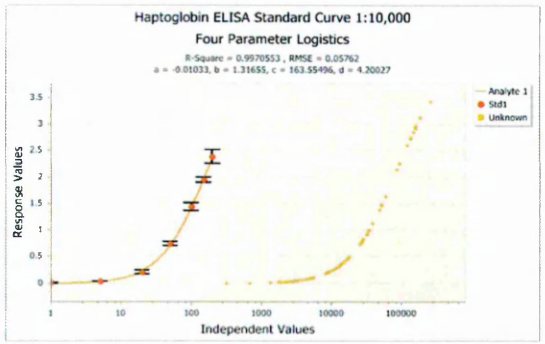
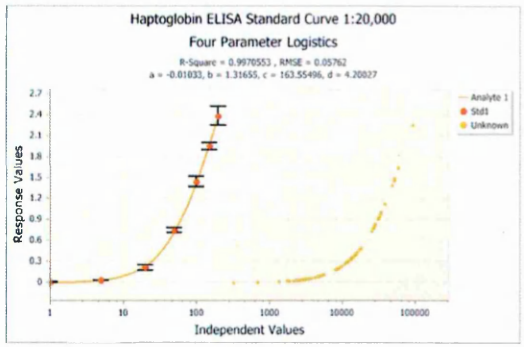
APPENDIX 1
Standard Curves

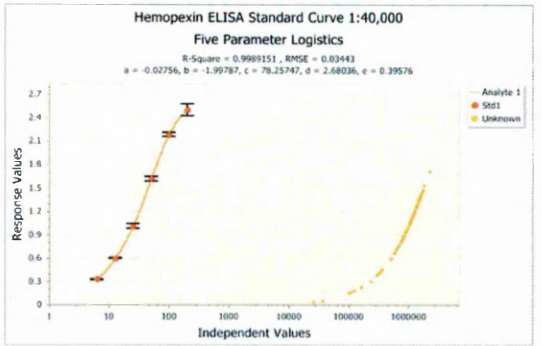




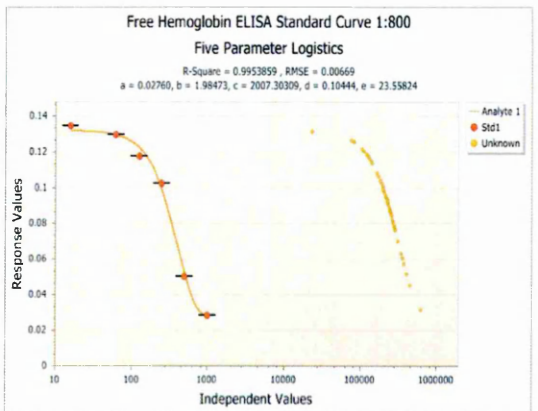


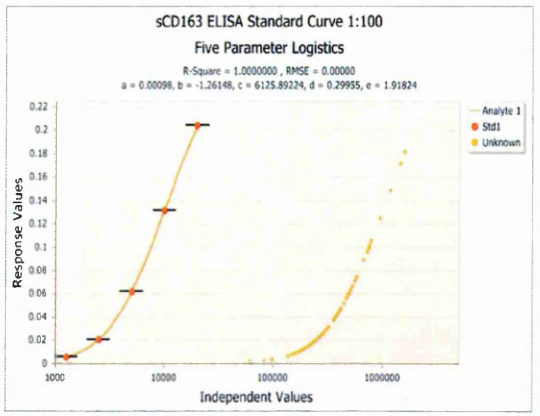


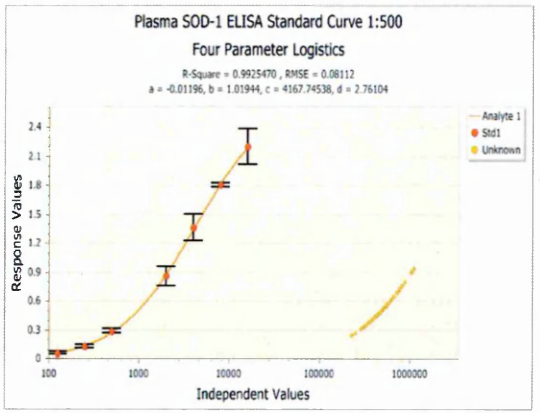


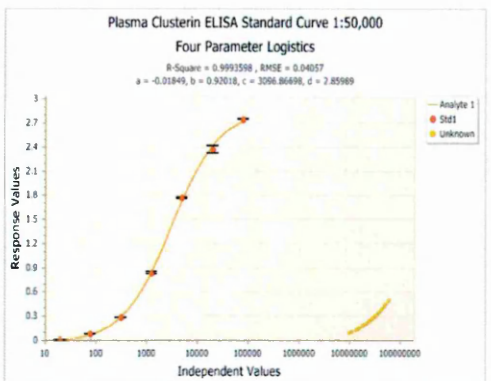


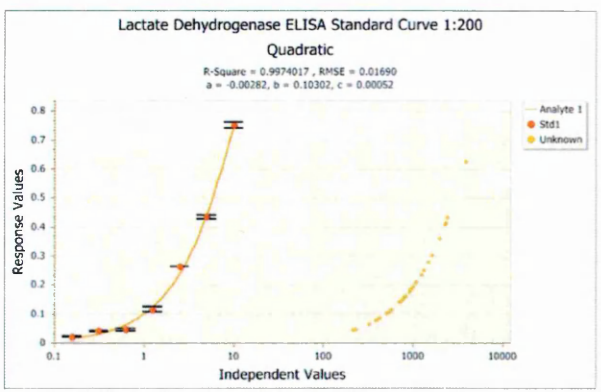
The Standard curves for the ELISA experiments as described in Chapter 2

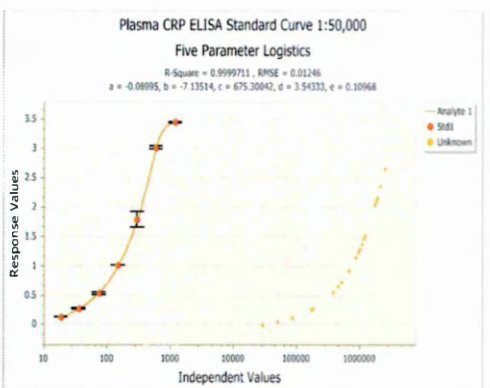












The Standard curves for the ELISA experiments as described in Chapter 2

APPENDIX 2

Known and unknown variants on *Hp* gene

##fileformat=VC							
S/N	#CHROM	POS	INFO	REF	ALT	Known Variant ID	Unknown Variant
1	16	72085496	AF=0.00166;AN=604;AC=1;DP=26217	G	T		✓*
2	16	72085561	AF=0.82768;AN=766;AC=634;DP=143688	T	A	rs9924964 *§	
3	16	72085704	AF=0.00244;AN=818;AC=2;DP=249053	TTTTC	T	rs551013110 *	
4	16	72085905	AF=0.00118;AN=846;AC=1;DP=431697	T	G		✓*
5	16	72086301	AF=0.00223;AN=896;AC=2;DP=857692	G	A	rs543817830	
6	16	72086321	AF=0.00787;AN=890;AC=7;DP=872489	T	C	rs112925814 *	
7	16	72086460-72086462	AF=0.19080;AN=870;AC=166;DP=618183	T	TTC		✓*
8	16	72086469-72086476	AF=0.18029,0.00120,0.00120;AN=832;AC=150,1,1;DP=384892	A...C	T..C,A..C,A..T		✓
9	16	72086534	AF=0.83269;AN=1034;AC=861;DP=797163	A	T	rs7201866 §	
10	16	72086555	AF=0.65800,0.00482;AN=1038;AC=683,5;DP=784895	T	C	rs7203426 $	
11	16	72086588	AF=0.05263,0.00195;AN=1026;AC=54,2;DP=214213	TTTTTTT	ATTTTTT,ATTTTTA		✓
12	16	72086741	AF=0.62882,0.02576;AN=1048;AC=659,27;DP=1862916	GCCTGA	GCCTGG,ACCTGG		✓
13	16	72086817	AF=0.00096,0.00096;AN=1046;AC=1,1;DP=1914582	C...G	C...A,T...G		
14	16	72087058	AF=0.16794;AN=1048;AC=176;DP=2346063	T	C	rs28639994	
15	16	72087058	AF=0.16794;AN=1048;AC=176;DP=2429727	T	C	rs555601020	
16	16	72087075	AF=0.01437;AN=1044;AC=15;DP=1911329	CT	C	rs555601020	
17	16	72087192	AF=0.00095;AN=1048;AC=1;DP=2600329	G	C	rs532682715	
18	16	72087307	AF=0.65774,0.00096;AN=1046;AC=688,1;DP=2482792	G	A		✓ $, $
19	16	72087324	AF=0.00095;AN=1048;AC=1;DP=2650104	G	A		✓
20	16	72087607	AF=0.00096;AN=1046;AC=1;DP=2552126	C	A	rs555848457	
21	16	72087773	AF=0.00095;AN=1048;AC=1;DP=2859485	AC	A	rs545957124	
22	16	72088014	AF=0.00095;AN=1050;AC=1;DP=2506763	A	C	rs545957124	
23	16	72088159	AF=0.00383;AN=1044;AC=4;DP=1404313	A	G	rs545957124	
24	16	72088227	AF=0.11905;AN=1050;AC=125;DP=1095565	T	C	rs5466 §	
25	16	72088280	AF=0.00478;AN=1046;AC=5;DP=851094	C	T	rs5467	
26	16	72088379	AF=0.00096;AN=1040;AC=1;DP=1129556	C	A	rs763451870	
27	16	72088418	.F=0.43919,0.17375,0.00386;AN=1036;AC=455,180,4;DP=128041	TGAC	AGAC,TGAG,TTAC		✓ $
28	16	72088461	AF=0.13953;AN=1032;AC=144;DP=1499372	A	C	rs5471 § $	
29	16	72088467	AF=0.47287;AN=1032;AC=488;DP=1551173	A	G	rs5472 $	
30	16	72088652	AF=0.47490,0.00193;AN=1036;AC=492,2;DP=1694323	CGGGC	TGGGC,TGGGG		✓
31	16	72088675	AF=0.65370;AN=1028;AC=672;DP=1899058	T	C	rs4788458	
32	16	72088684	AF=0.00097;AN=1036;AC=1;DP=1897282	G	C		✓
33	16	72088930	AF=0.00097;AN=1028;AC=1;DP=1156433	A	G		✓
34	16	72088948	AF=0.16862;AN=1026;AC=173;DP=978486	G	A	rs35184823	
35	16	72088964	AF=0.47173;AN=1026;AC=484;DP=1060759	C	T	rs8062041	
36	16	72088969	AF=0.00097;AN=1028;AC=1;DP=921685	G	C	rs146982445	
37	16	72088990	AF=0.00194;AN=1032;AC=2;DP=1032856	T	C	rs188894747	
38	16	72089044	AF=0.49902;AN=1024;AC=511;DP=703424	CT	C	rs576218458	✓
39	16	72089088	AF=0.00097;AN=1036;AC=1;DP=1201348	C	T		✓
40	16	72089165	AF=0.66344;AN=1034;AC=686;DP=1304135	G	A	rs2236765	
41	16	72089225	AF=0.00578;AN=1038;AC=6;DP=1289164	G	A	rs142176420	
42	16	72089246	AF=0.17087,0.00097,0.00194;AN=1030;AC=176,1,2;DP=795334	CCA....AC	TCA...AC,CCA...AA,CCA...AC		✓
43	16	72089312-72089312	AF=0.00096;AN=1042;AC=1;DP=1360862	C	A		✓

List of genetic variations (indels and SNPs) found in the sequenced Hp gene. S/N, Serial Number; #CHROM, Chromosome; POS, Position; INFO, Information; REF, Reference allele; ALT, Alternative allele; AF, Allele Frequency; AN, Total number of allele in called genotypes; AC, allele count in genotypes, for each ALT allele, in the same order as listed; DP, Depth of coverage, read depth at the given position. * = Deletions that show association with disease as shown in tables 6.1; § = Variants that show association with the disease as shown in tables 6.2; $ = Variants that show association with levels as shown in table 6.3.

	##fileformat=VCFv4.0						
S/N	#CHROM	POS	INFO	REF	ALT	Known Variant ID	Unknown Variant
44	16	72089740	AF=0.65504;AN=1032;AC=676;DP=524166	A	G	rs2070937	
45	16	72089451	AF=0.65957;AN=1034;AC=682;DP=1155201	T	C	rs12917999	
46	16	72089769	AF=0.00288;AN=1042;AC=3;DP=439512	A	G	rs72550714	
47	16	72089958	AF=0.16424;AN=962;AC=158;DP=221098	A	ATGTG	rs148260617	
48	16	72089973	AF=0.00706,0.16734;AN=992;AC=7,166;DP=432048	TGT	T,TGTGTGT		✓
49	16	72090052	AF=0.00195;AN=1028;AC=2;DP=1055455	C	G	rs78413891	
50	16	72090056	AF=0.00487;AN=1026;AC=5;DP=1054101	G	A	rs192501772	
51	16	72090177	AF=0.16699;AN=1018;AC=170;DP=1083525	C	G	rs77507186	
52	16	72090186	AF=0.00489;AN=1022;AC=5;DP=1038539	T	G	rs183338750	
53	16	72090413	AF=0.00095;AN=1048;AC=1;DP=910404	T	A		✓
54	16	72090450	AF=0.00382;AN=1046;AC=4;DP=783417	C	T	rs371786946	
55	16	72090626	AF=0.00288;AN=1042;AC=3;DP=410075	G	A	rs35461039	
56	16	72090656	AF=0.00096;AN=1044;AC=1;DP=410932	T	C		✓
57	16	72090666	AF=0.00192;AN=1042;AC=2;DP=413353	C	G	rs577584801	
58	16	72090685	AF=0.00096;AN=1044;AC=1;DP=421951	A	G	rs112957470	
59	16	72090785	AF=0.83109;AN=1042;AC=866;DP=437898	C	G	rs113760890	
60	16	72090931	AF=0.00192;AN=1042;AC=2;DP=880801	TTTA	T		✓
61	16	72090961-72091054	AF=0.00096;AN=1038;AC=1;DP=698594	C...T	A...T		✓
62	16	72091065	AF=0.00192;AN=1044;AC=2;DP=1696765	C	T		✓
63	16	72091102	AF=0.01346;AN=1040;AC=14;DP=1822536	TG	CA	and rs74621317	
64	16	72091152	AF=0.02885;AN=1040;AC=30;DP=1624981	A	G	rs651709	
65	16	72091405	AF=0.00678;AN=1032;AC=7;DP=918593	C	T	rs572762019	
66	16	72091412	AF=0.00097;AN=1032;AC=1;DP=951356	C	T	rs539869192	
67	16	72091565	AF=0.32046;AN=1036;AC=332;DP=1439359	G	A	rs74697591	
68	16	72091593	AF=0.07324;AN=1024;AC=75;DP=1325507	C	T	rs2550042	
69	16	72091598	AF=0.00714;AN=980;AC=7;DP=1318240	GG	TA		✓
70	16	72091602	AF=0.21673,0.01411;AN=992;AC=215,14;DP=1308569	CT	TT,TC		✓
71	16	72091614	AF=0.00413,0.00103;AN=968;AC=4,1;DP=1386118	C	T,G	rs2550041	
72	16	72091648	AF=0.53731;AN=938;AC=504;DP=1440558	A	G	rs664134	
73	16	72091683	AF=0.00202;AN=990;AC=2;DP=1380956	G	A		✓
74	16	72091808	AF=0.26353;AN=998;AC=263;DP=1690499	C	A	rs2259205	
75	16	72091819	AF=0.25051,0.00303;AN=990;AC=248,3;DP=1626712	AC	GC,GT	rs2259204	✓
76	16	72091908	AF=0.00102;AN=980;AC=1;DP=1460308	C	T		✓
77	16	72092017	AF=0.64384;AN=876;AC=564;DP=1539822	A	T	rs2070938	
78	16	72092137	AF=0.00111;AN=904;AC=1;DP=1260573	T	A		✓
79	16	72092157	AF=0.00111;AN=902;AC=1;DP=1162410	C	T	rs772006915	
80	16	72092384	AF=0.00299;AN=1004;AC=3;DP=605267	A	G	rs566471516	

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	##fileformat=VCFv4.0						
S/N	#CHROM	POS	INFO	REF	ALT	Known Variant ID	Unknown Variant
81	16	72092390	AF=0.28984;AN=1004;AC=291;DP=601442	C	G	rs3794693	
82	16	72092509	AF=0.71624;AN=1022;AC=732;DP=615371	C	G	rs56139130	
83	16	72092685	AF=0.00194;AN=1032;AC=2;DP=1459364	C	T		✓
84	16	72092685	AF=0.00194;AN=1032;AC=2;DP=1459364	C	T		✓
85	16	72092826	AF=0.01856,0.02680;AN=970;AC=18,26;DP=2340667	CA	TG,TT	rs374805959	✓
86	16	72092980	AF=0.00110;AN=908;AC=1;DP=1551454	C	T		✓
87	16	72093024	AF=0.00110;AN=910;AC=1;DP=1403414	T	C		✓
88	16	72093029	AF=0.00111;AN=900;AC=1;DP=1266935	CA	TG	rs1140430	
89	16	72093033	AF=0.00111;AN=900;AC=1;DP=1250721	G	A	rs200877317	
90	16	72093094	AF=0.19828;AN=1044;AC=207;DP=1022937	G	GCTGAGCA	rs200877317	
91	16	72093127	AF=0.00096;AN=1046;AC=1;DP=1418253	C	T	rs570083636	
92	16	72093202	AF=0.00669;AN=1046;AC=7;DP=1684389	G	A	rs72563793	
93	16	72093406	AF=0.00191;AN=1048;AC=2;DP=1733961	C	A		✓
94	16	72093498	AF=0.00096;AN=1046;AC=1;DP=1630313	T	A		✓
95	16	72093535	AF=0.01916;AN=1044;AC=20;DP=1491664	C	T	rs142781484	
96	16	72093755	AF=0.00095;AN=1050;AC=1;DP=968296	G	C	rs569377878	
97	16	72093764	AF=0.00096;AN=1040;AC=1;DP=978726	T	A		✓
98	16	72093776	AF=0.00096;AN=1042;AC=1;DP=1018597	C	T		✓
99	16	72093779	AF=0.00096,0.00096;AN=1038;AC=1,1;DP=1031421	C	T,A		✓
100	16	72093786	AF=0.00096;AN=1038;AC=1;DP=1012519	TG	AT		✓
101	16	72093790	AF=0.00096;AN=1038;AC=1;DP=947960	AGAAAAGTGG	A		✓
102	16	72093830	AF=0.00192;AN=1040;AC=2;DP=1117979	T	A		✓
103	16	72093834	AF=0.00192;AN=1040;AC=2;DP=1063828	AAACAG	A		✓
104	16	72093883	AF=0.01346;AN=1040;AC=14;DP=1257954	G	A	rs145040577	
105	16	72093890	AF=0.00096;AN=1040;AC=1;DP=1359469	G	A	rs599831	
106	16	72094118	AF=0.01062;AN=1036;AC=11;DP=2031179	A	T	rs375137689	
107	16	72094132	AF=0.00772;AN=1036;AC=8;DP=2105409	T	G	rs560865345	
108	16	72094285	AF=0.00096;AN=1046;AC=1;DP=2383338	C	T	rs776435847	
109	16	72094408	AF=0.00191;AN=1048;AC=2;DP=2647507	T	C	rs776302022	
110	16	72094573	AF=0.00096;AN=1046;AC=1;DP=2596137	T	C	rs111837860	
111	16	72094591	AF=0.00191;AN=1046;AC=2;DP=2565149	T	C	rs5476	
112	16	72094654-72094682	AF=0.00864,0.00192,0.00192;AN=1042;AC=9,2,2;DP=1837386	C..A	T..A,C..G,C..A		✓
113	16	72094678	AF=0.00287;AN=1046;AC=3;DP=2680950	T	C	rs594359	
114	16	72094682	AF=0.00192;AN=1044;AC=2;DP=2636826	A	G	rs470428	
115	16	72094976	AF=0.00581;AN=1032;AC=6;DP=1005813	T	C	rs554186466	
116	16	72095132	AF=0.00676;AN=1036;AC=7;DP=772910	T	C		✓
117	16	72095239	AF=0.00872;AN=1032;AC=9;DP=623404	AG	A	rs113234131	
118	16	72095312	AF=0.17282;AN=1030;AC=178;DP=493605	CTC	TTT		✓

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